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# SOME NEW CONSTITUENTS OF MILK.\*

SECOND PAPER.

## THE DISTRIBUTION OF PHOSPHATIDES IN MILK.

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(From the Laboratory of the Connecticut Agricultural Experiment Station,  
New Haven.)

(Received for publication, October 3, 1916.)

Having recently shown<sup>1</sup> that the alcoholic washings of the coagulated protein obtained by boiling the filtrate from casein of cow's milk yield to alcohol a small quantity of monamino- and diamino-phosphatides, we have now examined the alcoholic washings of casein and have found that these likewise contain a small amount of similar, if not identical phosphatides. We have also extended our investigation to the other parts of milk in order to learn as much as possible about the distribution of phosphatides in this important food.

The results of this examination have shown that when the filtrate from the heat-coagulable proteins is neutralized to phenolphthalein with sodium hydroxide a precipitate is produced which consists of about one-third protein and two-thirds calcium phosphate. This precipitate contains about the same percentage of phosphatides as does the coagulum produced by boiling the acid filtrate from the casein. Since the coagulum weighs about seven times as much as the neutralization precipitate, a much larger absolute quantity of phosphatide separates from the milk serum with the coagulum than with the neutralization precipitate. Since the "lecithalbumin" in the yolk of hen's eggs<sup>2</sup> yields about

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>1</sup> Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1915, xxi, 539.

<sup>2</sup> Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 413.

25 per cent of phosphatides it is evident that the relatively small amount of such substances obtained from the neutralization precipitate does not indicate the presence of any considerable amount of a similar lecithalbumin in the latter.

The solids left by evaporating the filtrate from the neutralization precipitate, which are very nearly free from fat and protein, were extracted with chloroform and afterwards with alcohol, but only a trace of substance insoluble in acetone was detected in the small amount of material soluble in these solvents. Since we have previously shown that butter fat contains only minute traces of phosphorus we can now conclude that all of the phosphatides present in cow's milk are associated with protein.

#### EXPERIMENTAL.

The alcoholic washings of large quantities of the casein of cow's milk which has been prepared in this laboratory were saved and from time to time worked up in order to recover from them whatever they might contain. In order to discover substances soluble in strong alcohol the washings were concentrated to small volume, the residue was treated with absolute alcohol, and the mixture again concentrated. After repeating this process several times and removing the insoluble substances by filtration a relatively concentrated solution was obtained which was practically water-free.

The weight of substance, thus soluble in alcohol, was determined by evaporating an aliquot part. Another part of the remainder was evaporated to remove the alcohol and the oily residue was dissolved in ether, filtered clear, and the filtrate poured into acetone. When the abundant precipitate which resulted was again treated with ether a part failed to dissolve. This had the white color and characteristic behavior of the diamminophosphatide described in our first paper of this series. Without filtering, the ether solution was poured into acetone and the precipitate washed with acetone and dried to constant weight *in vacuo* over sulfuric acid. When this was oxidized by heating with sulfuric acid and ammonium nitrate it was found to contain 3.53 per cent of phosphorus, or just about the proportion to be expected for a mixture of the diamino- and monaminophosphatides previously

obtained from the alcoholic washings of the lactalbumin.<sup>1</sup> The alcoholic washings of three lots of casein, which thoroughly air-dried weighed 56,752, 7,560, and 10,460 gm., yielded crude preparations of two mixed phosphatides equal to 0.033, 0.043, and 0.039 per cent.

Milk was freed from nearly all its fat by centrifugation, from casein by the careful addition of hydrochloric acid and filtration, and from coagulable proteins by boiling the *clear* filtrate from the casein for 2 or 3 minutes and filtering. 10 liters of the filtrate from the coagulum were cooled and then made very nearly neutral to litmus by adding 100 cc. of water containing 9.5 gm. of sodium hydroxide, whereby a voluminous precipitate was produced. After standing over night the somewhat turbid solution was syphoned off and the precipitate, I, collected as a gelatinous cake by centrifuging. To the slightly acid solution decanted from this cake 74 cc. of water, containing 7.04 gm. of sodium hydroxide, were added, whereby it was rendered faintly alkaline to phenolphthalein. The precipitate, II, thus produced, was filtered out, removed from the paper, and freed from adhering solution by centrifuging. The clear filtered solution was treated with 1,440 cc. of water containing 2 gm. of calcium hydroxide, until no more precipitate was produced. The precipitate, III, was filtered out and freed from solution by centrifuging.

The slightly alkaline filtrate was neutralized to litmus by adding 0.045 gm. of hydrochloric acid dissolved in considerable water. The whole was then evaporated on a steam bath at less than 85°. During most of the time the solution was evaporating the temperature was about 50°. The residue was ground, air-dried at a low temperature, and then *in vacuo* over sulfuric acid to a constant weight of 404 gm.

### *Precipitate I.*

Previous experiments have shown that the precipitate produced by neutralizing the milk serum obtained after removing casein and heat-coagulable proteins consists chiefly of calcium phosphate and protein. Since phosphatides are frequently associated with protein, we extracted Precipitate I, while still moist, eight successive times with absolute alcohol, care being taken to bring the substance into contact with the alcohol by passing it each time



through fine silk bolting cloth. After each treatment the solid was separated by centrifugation, and after the eighth extraction, when alcohol-soluble matters had been practically all removed, it was dried *in vacuo* over sulfuric acid. Thus prepared it formed a white powder which weighed 14.19 gm.

Analysis showed it to contain:

		Dried at 110°.
	per cent	per cent
Loss on drying at 110°.....	11.40	
N.....	2.69	3.04
P.....	11.30	12.75
Ca.....	21.88	24.70
Protein ( $N \times 6.38$ ).....	17.16	19.40
Ash.....	61.22	69.10

From these results the following have been calculated.

	per cent
Ca phosphate ( $P \times 5$ ).....	63.8
Protein ( $N \times 6.38$ ).....	19.4
Undetermined inorganic.....	5.3
Undetermined organic and water not lost at 110°.....	11.5
	<hr/> 100.0

The calcium and phosphorus are in the proportions required for  $Ca_3P_2O_8$  and are equal to 63.8 per cent of this substance in the material dried at 110°. This is 5.3 per cent less than the total ash, showing Precipitate I to contain other forms of inorganic matter. The nitrogen is equal to 19.4 per cent of protein, leaving 11.5 per cent of undetermined matters.

The united alcoholic extracts were concentrated *in vacuo* and the residue, which consisted largely of crystalline lactose,<sup>3</sup> was extracted with warm absolute alcohol and the insoluble matter filtered out. The alcoholic extract was concentrated *in vacuo* and the residue was again treated in the way just described. The matter insoluble in alcohol was soluble in water and was rejected

<sup>3</sup> This had probably been adsorbed by the very voluminous precipitate and rendered soluble again by the dehydrating action of the alcohol.

as consisting of lactose and inorganic salts. The residue which was left on evaporating the last alcohol solution was treated with alcohol and ether, and water-soluble substances were removed by adding water and shaking out with ether. The aqueous layer was repeatedly shaken out with ether and the united ether solutions were next washed several times with water and then evaporated. A thick oily residue remained which weighed 0.1386 gm. and was perfectly soluble in ether

### *Precipitate II.*

This substance when extracted with absolute alcohol, in the same way as was Precipitate I, and dried *in vacuo* over sulfuric acid weighed 17.66 gm.

Analysis showed it to contain:

		Dried at 110°.
	<i>per cent</i>	<i>per cent</i>
Loss on drying at 110°.....	12.20	
N.....	1.75	1.99
P.....	11.48	13.07
Ca.....	21.25	24.19
Protein ( $N \times 6.38$ ).....	11.17	12.72
Ash.....	60.56	69.00

From these results the following have been calculated.

	<i>per cent</i>
Ca phosphate ( $Ca \times 2.58$ ).....	62.5
Protein ( $N \times 6.38$ ).....	12.7
Undetermined inorganic.....	6.5
Undetermined organic and water not lost at 110°.....	18.3
	<hr/> 100.0

The calcium and phosphorus are here also in very nearly the proportions required for  $Ca_3P_2O_8$ , the phosphorus being slightly in excess. The amount of calcium phosphate equivalent to the calcium is equal to 62.5 per cent of the substance dried at 110°, or 6.5 per cent less than the total ash.

Treatment similar to that given to the alcohol extracts of Pre-

cipitate I yielded 0.1650 gm. of substance soluble in ether, equal to 0.3036 gm. of ether-soluble substance from the two neutralization precipitates.

*Precipitate III.*

When Precipitate III was subjected to the same treatment as I and II, 7.47 gm. of substance were obtained, which was analyzed with the following results:

		Dried at 110°.
	per cent	per cent
Loss on drying at 110°.....	17.01	
N.....	0.31	0.37
P.....	10.51	12.66
Ca.....	21.70	26.14
Protein ( $N \times 6.38$ ).....	1.98	2.38
Ash.....	59.89	72.16

From these results the following have been calculated.

	per cent
Ca phosphate ( $P \times 5$ ).....	63.3
Ca carbonate ( $CaCO_3$ ).....	4.0
Protein ( $N \times 6.38$ ).....	2.4
Undetermined inorganic.....	4.9
Undetermined organic and water not lost at 110°.....	25.4
	<hr/> 100.0

The phosphorus in this substance corresponds to 63.3 per cent of  $Ca_3P_2O_8$ , being 8.86 per cent less than the total ash. The ratio of calcium to phosphorus is a little greater than is required to form  $Ca_3P_2O_8$ .

The alcoholic extract of Precipitate III yielded 0.0775 gm. of oily substance which gave only a turbidity when its concentrated ethereal solution was poured into much acetone and cooled in an ice box over night.

The 404 gm. of residue, consisting chiefly of lactose and salts, obtained by evaporating the filtrate from the neutralization Precipitate III, were ground to a fine powder and extracted with 550

cc. of chloroform four successive times.<sup>4</sup> The extracts, when united and evaporated to dryness, left a residue weighing 0.2300 gm. This was soluble in ether, but its solution gave no turbidity when poured into acetone.

The residue, after thus extracting with chloroform, was extracted five successive times with absolute alcohol. The extracts were united, evaporated *in vacuo* to dryness, the residue dissolved in a little water and shaken out with ether. The ether solution when evaporated left 0.3765 gm., which was sparingly soluble in ether. This was treated with absolute ether, filtered, the insoluble residue washed with ether, and the ethereal solution evaporated to dryness. The residue, which weighed 0.2374 gm., contained only 0.16 per cent of phosphorus, was readily soluble in ether, and gave no turbidity when this solution was poured into acetone, hence practically no phosphatides were present.

The ether-soluble substance obtained from Precipitates I, II, and III together weighed 0.3811 gm., and from the 404 gm. of solids, obtained from the filtrate from Precipitate III, 0.4674 gm., a total of 0.8485 gm. Since the 10 liters of filtrate from the lactalbumin yielded 438.27 gm. of total solids, the substance soluble in ether was equal to 0.19 per cent of the latter, which is a little more than the 0.13 per cent previously obtained by direct and prolonged extraction of another sample of our "protein-free milk" with dry ether.

The ether-soluble substance from Precipitates I and II were united (equaled 0.3036 gm.), dissolved in a little ether, and poured into acetone. The resulting cloudy solution when kept on ice over night yielded a flocculent precipitate which was filtered out and washed with acetone. When this precipitate was treated with ether a turbid solution resulted, just like that of the mixed phosphatides obtained from the "lactalbumin," as described in the preceding paper<sup>1</sup> of this series. Since the entire amount of substance precipitated by acetone was very small its solution in ether was evaporated and the residue dried *in vacuo* over sulfuric acid. This weighed 0.0414 gm. and contained 3.57 per cent of phosphorus, an amount greater than that in the diaminophosphatides and somewhat less than in the monaminophosphatide.

<sup>4</sup> Cf. Brodrick-Pittard, N. A., *Biochem. Z.*, 1914, lxvii, 382.

This quantity of phosphatide is equal only to 0.009 per cent of the total solids, or to about 5 per cent of the total ether-soluble matter, obtained from the filtrate from the lactalbumin.

Since the phosphatides in the preceding experiments were extracted from solid substances which were for the most part insoluble in strong alcohol and ether, it is conceivable that some might escape solution and the amount of them be really greater than is here indicated. We accordingly separated the casein from 60 liters of centrifugated milk with dilute hydrochloric acid, and the coagulable proteins by boiling and filtering. The slightly acid solution was nearly neutralized to litmus with sodium hydroxide. Without removing the protein precipitated by thus neutralizing, the whole was evaporated at about 60°, to a volume of 5.5 liters. The concentrated solution from which much lactose had separated in fine crystals was poured into 4 liters of 92 per cent alcohol. The undissolved lactose and salts were sucked out, and to the 6.5 liters of filtrate 1.5 liters more alcohol were added. On standing, a little more lactose crystallized out of the deep yellow solution. The latter was concentrated under diminished pressure, more alcohol added, and this process continued as long as it was possible thus to separate products insoluble in absolute alcohol.

The final alcohol solution was then evaporated to a syrup, about 200 cc. of water were added, and the solution was shaken out five times with ether, containing a little alcohol. The ether solution was washed with water, evaporated, and the oily residue dissolved in 15 cc. of absolute ether and poured into 450 cc. of acetone. The precipitate which formed was redissolved in ether, filtered clear, concentrated to a small volume, and poured into much acetone. The precipitate, which weighed 0.1689 gm., contained 3.37 per cent of phosphorus.

The total solids contained in 60 liters of skimmed milk, after removing casein and the coagulable proteins, weighed 3,012 gm., of which the phosphatides formed 0.0056 per cent, or somewhat less than the 0.009 per cent found in the preceding experiment.

The distribution of phosphatides in the original milk can be stated as follows, assuming 1 liter of an average sample of milk to contain approximately the following quantities of the several fractions enumerated.

		Mixed phosphatides.
	gm.	gm.
Fat.....	35.0	None.
Casein.....	29.0	0.0102
"Coagulum".....	4.7	0.0124
Neutralization precipitate.....	4.6	0.0041
Lactose, salts, etc.....	55.0	None.
	128.3	0.0267

From these data it appears that the non-protein-containing fractions of milk contain no phosphatides and that the fractions which contain protein likewise contain phosphatides.

#### CONCLUSIONS.

Alcohol removes from milk casein, precipitated by dilute hydrochloric acid, about the same amount of phosphatides as was previously obtained from the "lactalbumin" obtained by heating the filtrate from the casein. Since the amount of casein is more than six times as much as the "lactalbumin," the proportion of phosphatide which it yields is correspondingly less.

The precipitate produced by treating skimmed milk, freed from casein and heat-coagulable proteins, with sodium hydroxide until neutral to phenolphthalein contains a very small amount of the same phosphatides and fatty substances that can be obtained from the alcoholic washings of the heat-coagulable proteins ("lactalbumin"). The non-protein fractions of fat-free milk contain at the most only minute traces of phosphatides. The total amount of phosphatides obtained from 1 liter of whole milk was equal to about 27 mg.

Phosphatides are intimately associated with the protein constituents of milk and possibly are combined with them as "lecithalbumins."



# THE ANTIGENIC PROPERTIES OF $\beta$ -NUCLEOPROTEINS.

By H. GIDEON WELLS.

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Otho S. A. Sprague Memorial Institute.)

(Received for publication, October 2, 1916.)

In a previous paper<sup>1</sup> I have discussed the antigenic properties of the so called "nucleoproteins" of tissues, both on the basis of the observations recorded in the literature and of personal experience. The following statement of Walter Jones<sup>2</sup> in reference to this class of substances, sometimes referred to as  $\alpha$ -nucleoproteins, expresses as completely and briefly as possible the correct status of the subject: "In reality 'nucleoprotein' means rather a 'method of preparation.'" My conclusions concerning their history and properties in immunological investigations were summarized as follows: Purified nucleins are not antigens, at least not in the ordinary sense, as none of their constituents (nucleic acid, histones, or protamines) is an antigen. Therefore any antigenic properties observed in "nucleoprotein" preparations are dependent on the protein component. There is no proof that such prepared nucleoprotein complexes are identical with compounds existing in the living cell, or that the proteins of these complexes are in any way specific for or characteristic of the cells from which they are obtained, or for the nucleoproteins themselves. Although results indicating a greater or less degree of organ or protein specificity have been described for these "nucleoprotein" preparations, they are contradicted by the negative results of other investigators. It is probable that the organ specificity demonstrated with such preparations by means of "saturation" methods is to be explained by the organ specificity which may be demonstrated by these methods with any organ extract or tissue juice. Chemical considerations and direct observations at present are against the view that the "nucleoproteins" exhibit any greater degree of organ specificity than cell proteins in general.

<sup>1</sup> Wells, H. G., *Z. Immunitätsforsch.*, 1913, xix, 599.

<sup>2</sup> Jones, W., *Nucleic Acids*, New York, 1914, 7.



Another class of material, of related but distinct character, has been isolated and described under the term " $\beta$ -nucleoproteins."<sup>3</sup> These have been found widely distributed by Jones and Rowntree.<sup>4</sup> Whereas the  $\alpha$ -nucleoproteins are extracted from tissues either by cold water, weak salt solution, or weak alkali, and precipitated with acetic acid, the  $\beta$ -nucleoproteins are extracted with boiling water and precipitated by acetic. Hence in the latter process all coagulable proteins are removed, a fact of much importance in considering the immunological reactions of the product, in view of the thermolability of most antigenic substances. While the  $\alpha$ -nucleoproteins represent salt-like compounds of nucleic acid with proteins of uncertain and apparently highly variable character, the  $\beta$ -nucleoproteins are compounds of guanylic acid with protein. The protein complex of the  $\beta$ -nucleoproteins has not been investigated, but in view of the fact that so few proteins are soluble in boiling water, it would seem probable that the possibility of variability and complexity of the protein side of these substances would be much less than with the  $\alpha$ -nucleoproteins.

I have tested the antigenic properties of three preparations of  $\beta$ -nucleoprotein by means of the anaphylaxis reaction. These were obtained by the method of Hammarsten from ox pancreas, ox spleen, and pig pancreas, these being selected in order that the species specificity and the organ specificity might be investigated. During their preparation all the necessary precautions were taken to avoid contaminating one protein with another, as required for anaphylaxis investigations.

The products from the pancreas both of ox and pig were nearly pure white, but that from the ox spleen was dark brown in color, in spite of the re-solution and repurification carried out six times with each preparation. All the preparations gave positive biuret, tryptophane, and Millon reactions, and on hydrolysis yielded a reducing sugar and a purine insoluble in weak ammonia, which gave a positive nitric acid reaction and a negative murexide test.

Anaphylaxis reactions were performed by sensitizing guinea pigs (175 to 200 gm. each) with 3 mg. of the proteins to be tested,

<sup>3</sup> Literature given by Jones.<sup>2</sup>

<sup>4</sup> Jones, W., and Rowntree, L. G., *J. Biol. Chem.*, 1908, iv, 289.

and after 3 weeks' interval injecting intraperitoneally 50 mg. of the protein dissolved in 5 cc. of 0.1 per cent NaOH.

The results are summarized in the following tables.

TABLE I.  
*β-Nucleoprotein of Beef Pancreas.*

No.	Sensitized with.	Reinjected with.	Result.	Protection reaction.
1	Beef pancreas.	Same.	Very severe.	
2	" "	"	Died, 105 min.	
3	" "	"	" 120 "	
4	" "	Beef serum.	Moderate.	Slight.
5	" "	" "	"	"
6	" serum.	" pancreas.	"	Severe.
7	" "	" "	"	"
8	" "	" "	Slight.	"
9	" pancreas.	Pig "	"	Slight.
10	" "	" "	"	"
11	" "	" "	"	"
12	" "	" "	"	"
13	" "	Beef spleen.		Moderate.
14	" "	" "		Slight.
15	" "	" "		Moderate.
16	" "	" "	Doubtful.	Slight.

TABLE II.  
*β-Nucleoprotein of Pig Pancreas.*

No.	Sensitized with.	Reinjected with.	Result.	Protection reaction.
1	Pig pancreas.	Same.	Moderate.	
2	" "	"	Severe.	
3	" "	"	Moderate.	
4	" "	Pig serum.	Severe.	
5	" "	" "	Moderate.	
6	" serum.	" pancreas.	Slight.	Severe.
7	" "	" "	"	"
8	" pancreas.	Beef pancreas.	Moderate.	Slight.
9	" "	" "	"	
10	" "	" "	"	Slight.
11	" "	" "	Slight.	
12	" "	" spleen.		
13	" "	" "		
14	" "	" "		
15	" "	" "		Slight.

TABLE III.,  
 $\beta$ -Nucleoprotein of Beef Spleen.

No.	Sensitized with.	Reinjected with.	Result.	Protection reaction.
1	Beef spleen.	Same.	Severe.	
2	" "	"	"	
3	" "	Beef serum.	Moderate.	Slight.
4	" "	" "	Severe.	Moderate.
5	" serum.	" spleen.	Doubtful.	Severe.
6	" "	" "		"
7	" spleen.	" pancreas.	Slight.	Slight.
8	" "	" "	"	Doubtful.
9	" "	" "	Moderate.	
10	" "	" "	"	
11	" "	Pig pancreas.	Slight.	Slight.
12	" "	" "	"	"
13	" "	" "	"	
14	" "	" "	"	

*Explanation of Tables.*—The term " $\beta$ -nucleoprotein" is omitted from the tables, only the name of the tissue from which they are derived being given. Under "Results" the terms employed will be found defined fully in a previous paper (Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1911, viii, 88). By "Protection-reaction" is meant the reaction that follows an injection of the protein used for sensitizing after an interval of 48 to 72 hours subsequent to the injection of the heterologous protein. If the heterologous protein has been closely enough related to the sensitizing protein and injected in sufficient amount, it should bind all the antibodies present and thus prevent any reaction following the second injection of the sensitizing protein; but in this series of experiments the amount of material available made it inexpedient to inject the heterologous protein in large enough amounts to insure complete saturation of antibodies. Hence the results in this column are not altogether conclusive, and they are not considered in estimating the final results of the experiments.

These experiments show that the  $\beta$ -nucleoprotein prepared from ox pancreas, and repurified six times, is an active antigen, producing very strong anaphylaxis reactions, comparable to those produced by most simple soluble proteins. This is of interest when compared with the results of anaphylaxis experiments with preparations of  $\alpha$ -nucleoproteins made without heat, for these entirely lose their anaphylaxis antigenic properties on repeated repurification. This observation carries with it a suggestion as to the nature of the protein radical in  $\beta$ -nucleoprotein,

for there are very few known proteins that will cause active anaphylaxis reactions after having been heated to boiling. These are casein,<sup>5</sup> ovomucoid,<sup>6</sup> and the so called proteoses of vegetable tissues.<sup>7</sup> Evidently the protein of  $\beta$ -nucleoprotein is entirely different from that of the  $\alpha$ -nucleoproteins, which is so readily denaturalized even by simple reprecipitation and re-solution by weak acetic and alkalis. It presumably is a non-coagulable protein, of which those mentioned above are examples; furthermore, it is a whole protein molecule, since the products of cleavage of protein molecules are not capable of causing anaphylaxis reactions. That this result with  $\beta$ -nucleoprotein from ox pancreas is not due to error in preparation is shown by the definite, although weaker, reactions obtained with the corresponding preparations from beef spleen and pig pancreas.

The crossed reactions between the three preparations of  $\beta$ -nucleoprotein and the blood serum of the same animals indicate the presence in these preparations either of a trace of serum proteins, or the coexistence of common antigenic groupings in the serum proteins and the nucleoproteins. There is no way of positively determining which is the correct explanation, since we have found evidence that chemically distinct but related proteins may give partial reactions with the antibodies for each other; *e.g.*, the alcohol-soluble proteins of rye, wheat, and barley.

We also find that beef pancreas  $\beta$ -nucleoprotein causes moderate reactions in animals sensitized either with beef spleen or pig pancreas  $\beta$ -nucleoprotein, which suggests that the protein in each preparation is chemically related although presumably not identical. However, the failure to secure reactions between pig pancreas and beef spleen, or to get reactions in pigs sensitized with beef pancreas and reinjected with beef spleen, leaves this point in some doubt. The activity of the spleen preparation was always lower than the others, which makes comparative results of doubtful value.

<sup>5</sup> Wells, *J. Infect. Dis.*, 1908, v, 449.

<sup>6</sup> Wells, *J. Infect. Dis.*, 1909, vi, 506; 1911, ix, 147. Elliott, C. H., *ibid.*, 1914, xv, 501.

<sup>7</sup> Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1915, xvii, 259.

for experimentation were guinea pigs maintained on an exclusive oat diet, since guinea pigs, particularly those of medium weight, show symptoms of the so called scorbutus within comparatively short periods of time. The animals were kept with food and water always present. No attempt was made to measure the daily consumption of oats, since the scattering of the food made this almost an impossibility in the majority of cases. The animals were weighed on alternate days, until the symptoms of malnutrition began to be manifest, and thereafter daily

TABLE I.  
*Exclusive Oat Diet.*

No.	Dietary days	Weight			Urea as mg. per 100 cc of fluid					Autopsy.
		Brain.	Muscle.	Test.	Blood.	Liver.	Heart.	Lung.	Muscle.	
A	4				43	24			112	Normal.
C	7				61	56	44	29	42	"
6	15	180	180	283	200	75			52	Marked hemorrhages.
5	18	755	755	155	115	38	61	52	85	Hemorrhages.
40	19	370	395	270	230					Very marked hemorrhages.
41	20	325	370	220	218					" " "
10	20	450	455	345	160	115	63	74	117	Marked hemorrhages.
B	24				165	52	114		43	Hemorrhages.
13	24	550	550	345	80	67		53	75	"
33	24	525	550	130	54					"
39	27	310	350	175	90					Marked hemorrhages.
D	30				159	127	88		122	Very marked hemorrhages.

for the most part. Liquids when fed were administered through a medicine dropper or small pipette. At the termination of the experiment the animals were placed under light ether anesthesia and bled from the carotid, the blood being collected in a casserole containing a small amount of neutral potassium oxalate to prevent clotting. Immediately thereafter the animals were carefully autopsied and the tissues to be studied were placed in weighed stoppered flasks containing 95 per cent alcohol. Blood urea was determined by the urease method of Van Slyke and Cullen, urea in the tissues by the method of Marshall and Davis.

The results obtained are shown in Table I, the experiments being tabulated in order, according to the duration of the experiment. In nearly every instance the animals lost weight during the first few days, then returned to the normal weight or in many instances to an increased weight. This weight was usually maintained for some days and was then followed by a rapid decline. With one exception (Guinea Pig 33), wherever autopsy showed hemorrhages of the bones, etc., described by Holst (1907) as typical,<sup>1</sup> there was observed a urea content of the blood and tissues, higher than normal. This increased urea content of the blood and tissues was most marked in the experiments with Guinea Pigs 40 and 41 in which after 19 and 20 days, analyses of the blood showed, respectively, 230 and 218

TABLE II.  
*Oats and Cabbage.*

No.	Duration.	Weight.			Urea as mg. per 100 cc. or gm.						Autopsy.
		Initial.	Max-imum.	Final.	Blood.	Liver.	Heart.	Lung.	Muscle.	Kidney.	
	days	gm.	gm.	gm.							
14	28	455	480	430	26	26	22	23	17	102	Normal. Well nourished.
7	31	600	600	600	26	26	23	27	25	50	" " "
32	42	315	390	390	46						" " "

mg. of urea per 100 cc. of blood. These animals showed on autopsy the most intense pathological changes seen in any of our experimental animals. The urea content of the tissues was variable, in some cases being of the same order of magnitude as the blood urea. In the greater number of animals with a high urea content in the blood, the tissues showed a urea content much lower than the blood and a lack of uniformity of distribution between the various tissues and organs observed in normal animals (Marshall and Davis, and Karr and Lewis). No relation between the urea content of the kidney and the condition observed at autopsy could be detected, both the highest and lowest figures (278 and 52 mg.) being found in the kidneys of animals with marked hemorrhages.

<sup>1</sup> We have in no case observed the loosening of the molar teeth described by Holst as typical.

Morgen and Beger have maintained that the condition resulting from an oat diet in rabbits is an acidosis which can be prevented by the administration of alkali. In Table IV are summarized the results of experiments on guinea pigs with diets of oats and sodium citrate. The citrate was administered in the form of a 15 per cent solution through a pipette or medicine dropper. The amounts fed per kilo of body weight were equal to or in most cases greater than the amounts of sodium bicarbonate fed by Morgen and Beger. After our experiments had shown that the addition of sodium citrate had little or no beneficial effect, the results of Funk (1916) already referred to were published, in which it was shown that guinea pigs failed to react to

TABLE V.  
*Fasting with or without Water.*

No.	Duration Days	Weight			Urea mg. per 100 cc. cc. gm.					Water Intake.	Autopsy.	
		Initial	Final	Loss	Blood	Liver	Heart	Lung	Muscle			
27	3	380	380	295	68	57	66	68	69	183	<i>Ad lib.</i>	Emaciated. Normal.
24	6½	590	590	455	93	90	117	90	101	208	"	" "
25	2	575	575	490	48	43	56	47	50	130	None.	" "
28	10	730	730	495	81	87	93		84	178	"	" "
26	7	490	490	340	43	46	42		43	57	30 cc.	" "
30	10	730	730	600	33						30 "	" "

the addition of alkali in the same manner as did rabbits. No mention is made of the amounts of bicarbonate fed. Inasmuch as our results confirmed those of Funk, we have confined our work to a short series of animals. The addition of sodium citrate to the oat diet had no marked effect in retarding the onset of the disease. In fact one of these animals (Guinea Pig 17) showed marked disturbances and pathological conditions at the end of 9 days, the shortest period in which we have observed such changes. An increased content of urea in blood and tissues was found as in the case of the animals on an exclusive oat diet.

Inasmuch as the food intake in experiments of this nature gradually diminishes with the development of the disease, the starvation factor must also be taken into account in any attempt

to explain these changes in the urea content of the organism. Although the food intake was lowered in these experiments, in no case was the animal allowed to fast completely more than 24 hours. When it was observed that no food had been consumed, the animals were killed shortly after. In most cases the period of complete fasting was less than 24 hours. Table V summarizes the results of a series of experiments on guinea pigs fasted for various lengths of time with or without water. In no case were typical hemorrhages observed on autopsy. The urea content of the blood and tissues was slightly higher than normal except in those cases where water was fed. Bang has shown that in rabbits these increases in the urea content of the blood after

TABLE VI.  
*Oats and Water per Os.*

No.	Duration.	Weight.			Blood urea as mg. per 100 cc.	Autopsy and other notes.
		Initial.	Maximum.	Final.		
	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		
37	17	380	420	325		30 cc. water <i>per os</i> daily. Hemorrhages.
34	24	595	625	480	66	20 cc. water <i>per os</i> daily. Marked hemorrhages.
36	27	340	360	220	126	20 cc. water <i>per os</i> daily. Very marked hemorrhages.
29	27	605	610	430	100	20 cc. water <i>per os</i> on last 3 days. Very marked hemorrhages.

fasting may be prevented by forced feeding of water. He maintains that in fasting the water intake is lowered so that the secretory power of the kidney is impaired and urea accumulates in the blood. Our experiments with guinea pigs confirm in a measure the work of Bang although the increases are by no means so marked as those reported by him for rabbits. The increases, even with no water present, over a fasting period of 10 days are less than those observed in the majority of cases of animals on an oat diet.

That fasting and diminished water intake are not the only factors involved in the increased urea content of the tissues is borne out by the experiments summarized in Table VI, in



Morgen and Beger have maintained that the condition resulting from an oat diet in rabbits is an acidosis which can be prevented by the administration of alkali. In Table IV are summarized the results of experiments on guinea pigs with diets of oats and sodium citrate. The citrate was administered in the form of a 15 per cent solution through a pipette or medicine dropper. The amounts fed per kilo of body weight were equal to or in most cases greater than the amounts of sodium bicarbonate fed by Morgen and Beger. After our experiments had shown that the addition of sodium citrate had little or no beneficial effect, the results of Funk (1916) already referred to were published, in which it was shown that guinea pigs failed to react to

TABLE V.  
*Feeding with or without Water.*

No.	Days	Weights			Urea as mg. per 100 cc. of fluid					Water intake	Autopsy.	
		Initial	Max. weight	Final	Blood	Liver	Heart	Lung	Muscle			
		gms.	gms.	gms.								
27	3	380	380	295	68	57	65	68	69	183	Ad lib.	Emaciated. Normal.
24	6	590	590	435	93	90	117	90	101	208	"	" "
25	2	575	575	490	45	43	56	47	50	130	None	" "
28	10	730	730	495	84	87	93		84	178	"	" "
26	7	490	490	340	43	46	42		43	57	30 cc.	" "
30	10	730	730	600	33						30 "	" "

the addition of alkali in the same manner as did rabbits. No mention is made of the amounts of bicarbonate fed. Inasmuch as our results confirmed those of Funk, we have confined our work to a short series of animals. The addition of sodium citrate to the oat diet had no marked effect in retarding the onset of the disease. In fact one of these animals (Guinea Pig 17) showed marked disturbances and pathological conditions at the end of 9 days, the shortest period in which we have observed such changes. An increased content of urea in blood and tissues was found as in the case of the animals on an exclusive oat diet.

Inasmuch as the food intake in experiments of this nature gradually diminishes with the development of the disease, the starvation factor must also be taken into account in any attempt

# THE ACTION OF SODIUM CITRATE AND ITS DECOMPOSITION IN THE BODY.

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## I.

The investigations of the earlier writers on the action of sodium citrate were confined chiefly to observations on toxicity. Mitscherlich (1) and later Vietinghoff-Scheel (2) determined the symptoms produced in animals by citrate, but it remained for the workers who followed them to inquire into its mode of action. This engaged the attention of Sabbatani (3), Loeb (4), Macallum (5), Auer (6), and Robertson and Burnett (7). Among the more recent investigations may be mentioned those of Gros (8) and of Salant and Hecht (9). Studies were also made on the changes produced when it is brought in contact with isolated tissues. According to Thunberg (10), citrate stimulates respiration in the isolated muscle of the frog. From the work of Battelli and Stern (11), it appears, on the other hand, that several isolated tissues of different animals, not including the blood, possess the power of oxidizing sodium citrate to carbon dioxide and water. Although it is well known that the carbonates in the urine are increased when citrate is fed, the extent of its oxidation in different animals has, to our knowledge, not been determined.<sup>1</sup>

The present report includes quantitative studies on the presence of citrate in the blood of animals when administered by mouth or subcutaneously, the rate of its disappearance from the circulation when injected intravenously, its oxidation in different animals and numerous observations on toxicity. The influence of citrate on coagulation also received some attention.

<sup>1</sup> According to unpublished results of Ohta, K. (*Biochem. Z.*, 1912, xlv, 481) citrates are incompletely destroyed in the body.



Weight of precipitate with Denigès' reagent.	Corresponding weight of dry sodium citrate.	Weight of precipitate with Denigès' reagent.	Corresponding weight of dry sodium citrate.
mg.	mg.	mg.	mg.
1.10	1.30	16.40	6.75
1.65	1.50	17.05	7.00
2.33	1.75	17.70	7.25
2.95	2.00	18.32	7.50
3.55	2.25	19.05	7.75
4.10	2.50	19.70	8.00
5.00	2.82	20.37	8.25
5.44	3.00	21.07	8.50
6.15	3.25	21.75	8.75
6.85	3.50	22.45	9.00
7.55	3.75	23.10	9.25
8.31	4.00	23.80	9.50
9.15	4.25	24.50	9.75
10.00	4.50	25.22	10.00
10.90	4.75	25.75	10.25
11.82	5.00	26.42	10.50
12.50	5.25	26.95	10.75
13.10	5.50	27.50	11.00
13.80	5.75	28.05	11.25
14.45	6.00	28.60	11.50
15.10	6.25	29.15	11.75
15.75	6.50	29.66	12.00

By means of the above procedure and by the use of the table, it was found possible to assay rapidly aqueous solutions of sodium citrate or citric acid, the error ordinarily being  $\pm 2$  per cent.

*B. Determination of Citrates in Urine.*—Some modifications were found necessary in citrate-containing urines. In fact, individual urines demanded variations of procedure which presented very little difficulty. The following plan was generally used.

A definite aliquot portion of the 24 hour urine,<sup>2</sup> that had previously been rendered acid with hydrochloric acid and filtered, was treated with a sufficient excess of 10 per cent barium acetate solution to insure an excess of barium ion, and followed by enough saturated barium hydroxide solution to render the mixture faintly alkaline. Three volumes of 95 per cent alcohol were then added.

<sup>2</sup> One-tenth of the 24 hour urine in the case of rabbits and cats was found a convenient portion. In the case of dogs a smaller aliquot was used.

*II. A Method for the Determination of Citrates in Blood and Urine.*

*A. Preliminary Experiments with Aqueous Solutions of Sodium Citrate.*—A method based on Denigès' test was employed for the determination of the citrate in the blood and urine. The following reagents were used.

1. Denigès' reagent was prepared by mixing in a 2 liter flask 48.5 gm. of red mercuric oxide with 500 cc. of water, 200 cc. of concentrated sulfuric acid being added very slowly with constant shaking. When the solution had cooled it was diluted with water to 1 liter in a volumetric flask and was filtered.

2. Syrupy phosphoric acid containing 85 per cent of ortho-phosphoric acid.

3. 3 per cent hydrogen peroxide.

4. 2 per cent potassium permanganate solution.

Exactly 0.7 cc. (about twenty drops) of Denigès' solution and as nearly as possible 0.13 cc. (about five drops) of phosphoric acid were added to 5 cc. of a citrate solution so chosen as to contain not more than 12 or less than 1 mg. of sodium citrate. The addition of reagents was facilitated by the use of 10 cc. burettes calibrated to 0.05 cc. The solution was heated to boiling and treated drop by drop with potassium permanganate solution until a slight but distinct pink color persisted even after continued heating. In the presence of citrates, a characteristic cloud, settling into a granular cream-colored precipitate, formed soon after the addition of the first drop of permanganate. The mixture was decolorized by means of three drops of the peroxide solution, heated to boiling, cooled, and Denigès' precipitate filtered on a weighed Gooch crucible, washed with 100 cc. of water, followed by 40 cc. of alcohol and finally with about 20 to 40 cc. of ether, and dried at 90–100°C. From the results obtained in repeated experiments under the exact conditions outlined above it became apparent that definite weights of sodium citrate yielded definite weights of Denigès' precipitate. The average weights of precipitate obtained from 1.3, 1.75, 2.5, 3, 4, 5, 7.5, 10, and 12 mg. of sodium citrate were used in plotting a curve, in which the weights of sodium citrate were used as abscissæ and the weights of Denigès' precipitate as ordinates. The appended table, which gives weights of precipitate corresponding to definite weights of sodium citrate, was prepared by the use of this graph.

the end-point being a persistent deep brown color or the usual potassium permanganate pink. By use of the table the amount of citrate (as sodium citrate) in the aliquot was found and the total amount of citrate excreted *per diem* was computed.

It has been stated that the aliquot taken for analysis must not contain over 10 mg. of sodium citrate (or its equivalent in citric acid). To ascertain this, it is well to run a rough preliminary test on a similar aliquot portion of the filtrate. Provided the potassium permanganate solution is rapidly and completely decolorized even after the addition of ten to twelve drops of this reagent, a larger amount of citrate may be suspected and a correspondingly smaller aliquot should be taken for the final analysis.

In a large number of tests with the urines of normal rabbits and cats, it was found that final aliquots representing one-twentieth of the total 24 hour urine did not respond to Denigès' test. In a few isolated cases a faint turbidity or negligible precipitate was noted. However, when small amounts of citrate were added to normal urines Denigès' test was invariably positive, provided more than 1 mg. of sodium citrate was present in the aliquot taken for analysis.

The following table gives the results of some analyses of normal urines to which definite amounts of sodium citrate had been added. Determinations were made on aliquot portions by the above method.

Volume of urine of normal rabbits.	Weight of anhydrous sodium citrate added.	Final aliquot.	Citrate found in aliquot by the method described.	Total citrate recovered.
cc.	mg.		mg.	mg.
50	100	$\frac{1}{20}$ of total urine.	5.38	107.6
50	100	$\frac{1}{20}$ " " "	5.01	100.2
50	100	$\frac{1}{20}$ " " "	5.14	102.8
50	100	$\frac{1}{20}$ " " "	4.82	96.4
50	200	$\frac{1}{20}$ " " "	8.55	213.7
50	200	$\frac{1}{20}$ " " "	8.37	209.3
50	200	$\frac{1}{20}$ " " "	4.10	205.0
50	200	$\frac{1}{20}$ " " "	4.35	217.5

7. *Determination of Citrates in Blood.*—15 cc. of blood were from an artery, vein, or directly from the heart into a graduated cylinder containing 75 cc. of 0.1 N acetic acid. The amounts of acetic acid employed varied, however, with the quantity of blood taken. The mixture was subsequently transferred to a Erlenmeyer flask, thoroughly shaken, and heated on a steam bath about an hour, until complete protein coagulation was observed. The mixture was then cooled, diluted to 100 cc., re-

turned to the flask, and thoroughly shaken with about 5 gm. of clean white sea sand to insure subdivision of the coagulated protein. The protein material was then in the form of a brown, creamy paste, readily filterable through a 12.5 cm. fluted filter paper, and yielding a clear nearly water-white filtrate. Exactly 60 cc. of this filtrate (corresponding to 9 cc. of blood) were treated with 10 cc. of a 10 per cent barium acetate solution, 80 cc. of 95 per cent alcohol, and just enough saturated barium hydroxide solution to render the mixture very faintly alkaline to litmus. The mixture was allowed to stand for an hour, or over night if very small amounts of citrate were suspected, shaken, and then centrifuged in successive portions in a 50 cc. centrifuge cup until the precipitate containing the citrate formed a compact layer on the floor of the cup from which the supernatant liquor could be readily decanted. The precipitated layer was treated and brought into intimate contact with 40 cc. of 50 per cent alcohol, and the suspension was again centrifuged. This operation was repeated until the supernatant liquid was free from chlorides.

After the supernatant liquors were removed as completely as possible by decantation, the residue was treated with five drops (0.125 cc.) of syrupy phosphoric acid, run in from a calibrated burette, and sufficient hot water (10 to 15 cc.) added to dissolve the precipitate. The solution was quantitatively transferred to a test-tube of about 200 cc. capacity, two or three glass beads were added, and the solution was cautiously evaporated over the free flame until a volume of about 8 cc. was reached. Care must be taken because of frothing. The solution was subsequently treated with twenty-five drops (0.80 cc.) of Denigès' reagent, described above, added from a calibrated 10 cc. burette. A precipitate, consisting in part of  $\text{BaSO}_4$ , formed, but remained partly suspended in finely divided form.

To this suspension was added a small amount of acid-washed purified talc, the mixture boiled for a few minutes, allowed to cool and to stand for about  $\frac{1}{2}$  hour, then quantitatively filtered by suction through a freshly prepared, acid-washed, asbestos-lined Gooch crucible into a test-tube  $6 \times \frac{3}{4}$  inches, the large test-tube being rinsed with about 5 cc. of water and the rinsings also filtered through the Gooch crucible. Unless the filtrate was clear and water-white, the filtration was repeated. The filtered solution was evaporated to 6 cc. and the analysis from this point on

was carried out just as described in the method for determining citrates in urine.

The foregoing method, however, is by no means quantitative and simply gives a very useful means of roughly approximating small amounts of citrate present in the blood. The following table clearly shows the limitations of the method and indicates that only about 75 per cent of the sodium citrate present in the blood can ordinarily be recovered.

Blood used.	Source.	Citrate added.	Citrate recovered.	
cc.		mg.	mg.	per cent
15	Dog.	None.	None.	
15	"	"	"	
15	"	"	"	
15	"	"	Very slight, positive test negli- gible.	
15	Rabbit.	3	2.60	87*
15	"	3	2.55	85*
15	"	5	3.00	60*
15	"	5	3.98	80*
15	"	5	3.75	75*
15	"	10	6.05	60*
15	"	10	6.80	68*
15	"	20	16.50	82
2	"	20	15.60	78
2	"	20	14.50	72
15	"	10	7.4	74
15	"	10	8.25	82.5
15	"	20	12.40	62
15	"	20	13.58	68
15	"	20	15.57	78
15	"	20	14.57	73
15	"	20	13.11	65.5
15	"	20	14.77	74
13	"	20	18.21	91
2	"	20	15.80	79
17	"	10	8.3	83
12	"	10	7.74	77
10	"	10	10.58	106
7.5	Dog	5	3.9—	78
Average.				+76

\* Time of analysis 48 hrs.



The causes of these wide discrepancies were not fully investigated, but it was apparent from the following experiment that they were at least in part due to the effect of protein coagulation. 15 cc. of blood were used, the protein coagulated as described above, 60 cc. aliquots were filtered off, and definite amounts of dry sodium citrate added to the aliquots. The results indicate that under these conditions citrates may be quantitatively determined with a fair degree of accuracy.

Sodium citrate added. mg.	Sodium citrate recovered. mg.
12	11.95
12	11.78
3	3.16

As other methods of removing protein, such as defibrinating blood, centrifuging off corpuscles, and using colloidal iron, proved unsatisfactory, we have used the above procedure in spite of its limitations.

### *III. The Presence of Citrate in the Blood after Subcutaneous Injection and When Given by Mouth.*

The experiments were carried out on rabbits, citrate being received subcutaneously by three. One was injected with 0.5 gm. per kilo and two others received 1.0 gm. per kilo. After an interval of 5 minutes 15 cc. of blood were withdrawn and tested for citrates, but this proved negative. Evidence of the presence of the salt was first obtained 15 minutes after its injection, about 2 mg. of citrate being the amount estimated in this quantity of blood. At the end of half an hour following the administration of citrate the amount increased to 8 or 9 mg., which remained stationary for a considerable time as the same results were obtained 1 and 2 hours later. Although much larger doses of the salt were given by mouth, the quantities detected in the blood were very small. In one experiment, in which 2 gm. of citrate per kilo were given, the amount present in the blood was 3.3 mg. per 15 cc., which was obtained 3 hours after administration. In another experiment in which 5 gm. of citrate per kilo were fed, the amount of citrate in 15 cc. of blood was slightly over 6 mg. after 3 hours. Tests made 1 and 2 hours after feeding citrate gave a faint reaction. Protocols are given below.

*Subcutaneous Injection.*

*Rabbit 1.*—Previous to the experiment the rabbit was fasted for about 2 days.

June 4, 1915. Weight 2,040 gm. 10 a.m. 2 gm. of urethane *per os* in 20 cc. of water. 11 a.m. Small amount of ether given. Cannula inserted into carotid artery. 11.14 a.m. 1 gm. of sodium citrate in 10 cc. of water injected subcutaneously into the abdominal tissue. Slight struggle. No marked symptoms. 11.19 a.m. 15 cc. of blood drawn (Sample 1). No citrate detected. 11.24 a.m. Very slight tremors. 11.30 a.m. 15 cc. of blood drawn (Sample 2). About 2 mg. of citrate detected. 11.44 a.m. 15 cc. of blood drawn (Sample 3). 3+ mg. of citrate detected.

Sample.	Time after injection.	Citrate recovered from 15 cc. of blood.
	<i>min.</i>	<i>mg.</i>
1	5	None.
2	16	About 2.
3	30	3+.

*Rabbit 2.*—Female, white; pregnant. Previous diet, oats and hay.

June 8, 1915. Weight 1,945 gm. 10.25 a.m. 19.2 cc. of 10 per cent citrate solution injected subcutaneously. 10.50 a.m. Cannula inserted into carotid artery. Ethyl chloride anesthesia. Tremors and dyspnea noted. 10.55 a.m. 15 cc. of blood drawn (Sample 1). 8 mg. of sodium citrate recovered. 11.20 a.m. Slight dyspnea, persisted. 11.24 a.m. Marked twitching. Muscular tremors. 11.55 a.m. Dyspnea. Salivation. Marked tremors. Local spasms. 15 cc. of blood drawn (Sample 2). 7.8 mg. of sodium citrate recovered. 1 p.m. 15 cc. of blood drawn (Sample 3). 8.3 mg. of sodium citrate recovered. 1.11 p.m. Marked dyspnea. 1.14 p.m. Respiration ceased, heart still beating. 1.16 p.m. Heart stopped.

*Autopsy.*—Heart dilated, soft, and injected. Kidneys normal. Gall bladder distended with light green bile.

Sample.	Time after injection.	Citrate recovered from 15 cc. of blood.
		<i>mg.</i>
1	30 min.	8
2	1½ hrs.	7.8
3	2½ "	8.3

*Rabbit 3.*—Male, white. Previous diet, oats and hay.

June 8, 1915. Weight 1,810 gm. 10.30 a.m. Received subcutaneous injection of 18 cc. of 10 per cent citrate solution. 10.35 a.m. Local anesthesia. 11 a.m. Tremors. Dyspnea. Defecation. 15 cc. of blood drawn

(Sample 1). 9.1 mg. of citrate recovered. 11.20 a.m. Slight dyspnea. 11.27 a.m. Muscular twitchings. 11.58 a.m. 15 cc. of blood drawn (Sample 2). 10.1 mg. of citrate recovered. Marked tremors. Gasping. Dyspnea. 12.05 p.m. Died.

*Autopsy.*—Heart injected and dilated. Kidney petechiated and soft. Liver normal. Gall bladder injected with bile.

Sample.	Time after injection.	Citrate recovered from 15 cc. of blood.
		mg.
1	30 min.	9.1
2	1 hr. 28 min.	10.1

### *Administration by Mouth.*

*Rabbit 4.*—Female, Belgian. Previous diet, oats and hay.

June 10, 1915. Weight 1,580 gm. 10.10 a.m. Received by mouth 32 cc. (2 gm. per kilo) of a 10 per cent sodium citrate solution, followed by 30 cc. of water. No symptoms. 11.10 a.m. Ethyl chloride anesthesia. Cannula inserted into carotid artery. 15 cc. of blood drawn. Faint test for citrate. 1.10 p.m. 15 cc. of blood drawn from carotid artery contained about 3.3 gm. of sodium citrate. No symptoms.

*Rabbit 5.*—Male, Belgian. Diet, oats and a little hay. Small amount of carrots fed on day preceding the administration of citrate.

June 16, 1915. Weight 1,280 gm. 9.40 a.m. Received by mouth 38.5 cc. (3 gm. per kilo) of a 10 per cent citrate solution. 11.35 a.m. No marked symptoms. Ethyl chloride anesthesia. Cannula inserted into carotid artery. 11.40 a.m. 15 cc. of blood drawn. 1 to 2 mg. of citrate recovered. Distinct increase in peristalsis. 1.30 p.m. Slight tremors. 2.30 p.m. 13.5 cc. of blood drawn (animal died during bleeding). 0.5 cc. of blood drawn from the heart. Very slight but distinct citrate test (about 2.2 mg. of citrate estimated).

*Rabbit 6.*—Female, Belgian. Previous diet, oats and hay.

June 14, 1915. Weight 1,350 gm. 10.30 a.m. Received by mouth 67.5 cc. (5 gm. per kilo) of 10 per cent citrate solution. 1.15 p.m. Paresis of extremities. 1.20 p.m. Cannula inserted into carotid artery. Ethyl chloride anesthesia. 1.25 p.m. 15 cc. of blood drawn. 6.05 mg. of citrate recovered. 3 p.m. Animal dead.

### *IV. The Disappearance of Citrate from the Circulation.*

Rabbits and dogs were used in these experiments. After removal of blood for control tests, sodium citrate was injected intravenously. Samples of the blood were then taken at various intervals after the salt was administered and quantitative determinations made. The tests showed that it disappeared rapidly

from the circulation. Thus, when 50 to 60 mg. of citrate per kilo were introduced into the ear vein of rabbits, the blood obtained within 3 to 5 minutes indicated that only small quantities were present. The total amounts as estimated in 15 cc. of blood did not exceed 25 mg., or about 10 to 15 per cent of the amount introduced.

In experiments made on dogs, approximately 100 mg. of sodium citrate per kilo were injected into the femoral vein. Analysis of the blood showed that its disappearance was rapid at first but was retarded later. About 60 to 70 per cent disappeared in 20 seconds and only 10 to 20 per cent disappeared during the next 5 to 10 minutes. Different individuals varied, however, in this respect. In one experiment appreciable amounts could still be found  $13\frac{1}{4}$  minutes after injection. The effect of repeated doses was also studied. When a second injection was made after an interval of 15 minutes, the citrate persisted much longer in the circulation. The quantities found in the blood at corresponding periods were also larger after the second injection. Protocols illustrating the results obtained are given below.

*Rabbit 7.*—Female, Belgian. Previous diet, oats and a small amount of hay.

Apr. 12, 1915. Weight 1,780 gm. 11.55 a.m. Ether administered. Canula inserted into carotid artery. 11.59 a.m. Injection of 4.8 cc. of 2.5 per cent citrate solution into ear vein. Dyspnea. 12.04 p.m. 15 cc. of blood drawn from carotid artery. Slight test for citrate. 2.3 mg. of sodium citrate estimated. 12.09 p.m. 15 cc. of blood drawn. Very faint positive test for citrate. 12.14 p.m. Animal bled to death.

*Dog 1.*—Female, black and white.

Apr. 29, 1915. Weight 6,900 gm. Condition good. 9.30 a.m. Received by mouth through stomach tube 3 gm. of chloretona in 30 cc. of 50 per cent alcohol. 9.45 a.m. Completely anesthetized. 10.33 a.m. Sample 1. 15 cc. of blood drawn from carotid artery. 10.37 $\frac{1}{2}$  a.m. 28 cc. of 2.5 per cent sodium citrate introduced into femoral vein in 2 minutes and 20 seconds. 10.37 $\frac{1}{2}$  a.m. Sample 2, 10.42 $\frac{3}{4}$  a.m., Sample 3, 10.47 $\frac{1}{2}$  a.m., Sample 4, each 15 cc. of blood drawn from carotid artery. 10.54 $\frac{1}{4}$  a.m. 28 cc. of 2 per cent sodium citrate introduced into femoral vein in 2 $\frac{1}{4}$  minutes. 10.55 a.m. Respiration good. 10.55 $\frac{1}{2}$  a.m. Sample 5, 10.59 $\frac{1}{2}$  a.m., Sample 6, 11.04 a.m., Sample 7, each 15 cc. of blood drawn from carotid artery. 11.08 a.m. Laparotomy. 25 cc. of urine drawn from bladder by means of a hypodermic syringe. 11.10 a.m. 48 cc. of 2.5 per cent sodium citrate injected through femoral vein. 11.12 a.m. Muscular tremors. Respiration stopped. Heart stopped.

Sample.	Amount of citrate in 15 cc. of blood.	Time after injection.
	<i>mg.</i>	
1	None.	Control.
2	6	20 sec. after first.
3	3	5 min. " "
4	2.5±	10 " " "
5	11.5	45 sec. " second.
6	7.4	5 min. " "
7	4.2	9½ " " "

*Dog. 2.*—Male, brown and white.

Apr. 26, 1915. Weight 6,550 gm. Chlorotone alcohol anesthesia. 1.57 p.m. Sample 1. 15 cc. of blood drawn from carotid artery. 2.01½ p.m. 26 cc. of 2.5 per cent sodium citrate injected into femoral vein. 2.02 p.m. Sample 2. 15 cc. of blood drawn from carotid artery. Respiration ceased immediately after blood was withdrawn, but returned in 1 minute and 5 seconds. 2.07 p.m. Sample 3, 2.10 p.m., Sample 4, each 15 cc. of blood drawn from carotid artery. 2.17½ p.m. 26 cc. of 2.5 per cent sodium citrate injected into femoral vein. Respiration ceased 45 seconds after injection was begun, but returned 45 seconds after its completion. 2.18 p.m., Sample 5, 2.23 p.m., Sample 6, 2.27 p.m., Sample 7, each 15 cc. of blood drawn from carotid artery. 2.33 p.m. 14 cc. of 2.5 per cent sodium citrate injected into femoral vein. Respiration stopped a few seconds after injection. Returned soon and increased in frequency in 4½ minutes. Apnea for 1½ minutes. Heart did not cease beating. 2.33 to 2.38 p.m. 34 cc. of 2.5 per cent sodium citrate injected into femoral vein. 3.04 p.m. 37 cc. of 2.5 per cent sodium citrate injected into femoral vein. Respiration slower. 3.04 to 3.06 p.m. 13 cc. of 2.5 per cent sodium citrate injected into femoral vein. 3.20 to 3.28 p.m. 50 cc. of 2.5 per cent sodium citrate injected into femoral vein. 3.44 to 3.47 p.m. 38 cc. of 2.5 per cent sodium citrate injected into femoral vein. 3.47 p.m. Respiration stopped. Heart still beating faintly. 3.50 p.m. Animal dead.

Sample.	Amount of citrate in 15 cc. of blood.	Time after injection
	<i>mg.</i>	
1	None.	Control.
2	7.9±	10 sec. after first.
3	2.5±	5½ min. " "
4	2.0±	10 " " "
5	13.3	50 sec. " second.
6	4.6	5½ min. " "
7	3.4	9½ " " "

The rapid disappearance of citrate from the circulation, as observed in herbivorous as well as in carnivorous animals, furnished a striking illustration of the protective action of the body against poisonous substances. As was shown by Gros (8) and by Salant and Hecht (9) this salt is a powerful cardiac depressant. The mechanism involved in ridding the blood of citrate probably includes several factors, chief among them being oxidation. Elimination, though probably playing a subordinate rôle, may also be regarded as important. Although oxidation in the blood would seem to be improbable, according to the results obtained by Battelli and Stern (11), it seemed desirable to test the validity of their conclusions. Experiments were therefore carried out in which blood drawn from rabbits and dogs was defibrinated, mixed with varying amounts of sodium citrate, and analyzed immediately or within 22 to 95 hours. The amounts recovered were approximately the same in all cases. The length of time allowed to elapse between the treatment and analysis had no effect on the amount of citrate recovered, nor were the results modified by temperature ranging from 10–39° (after 2 to 3½ hours) or in the presence of oxygen.

As shown in the following tables, the amounts recovered after 3 to 3½ hours when kept at temperatures of 36–39°C., a constant stream of oxygen being allowed to pass through, were the same as when the blood was kept at a temperature of 10°C. without oxygen.

#### V. The Effect of Blood on Sodium Citrate.

*Experiment with Oxygenated Rabbit Blood.*—A mixture of 19 cc. of blood and 1 cc. of 0.9 per cent sodium chloride containing 200 mg. of sodium citrate was oxygenated at 36–38°C. for about 3½ hours. At the end of this period the blood was bright red, unhemolyzed, and had not clotted. A sample representing one-twentieth of the total amount was taken for analysis.

Amount of citrate theoretically present.....	10 0 mg.
“ “ “ recovered .....	7 2 “

*Experiment with Oxygenated Dog Blood.*—A mixture of 19 cc. of blood and 1 cc. of water containing 200 mg. of sodium citrate was oxygenated at about 39°C. for about 3 hours. At the end of this

period the blood was red, unhemolyzed, and had not clotted. A sample representing one-twentieth of the total amount was taken for analysis.

Amount of citrate theoretically present..... 10.0 mg.  
 " " " recovered..... 7.9 "

*Experiment with Rabbit Blood to Which Sodium Citrate Was Added.*—To 15 cc. of fresh undefibrinated blood were added about 150 mg. of dry sodium citrate. The blood was kept in the ice chest at a temperature of about 10°C.

Time after blood was withdrawn.	Remarks.	Amount of citrate per 1 cc. of blood.
hrs.		mg.
22	No coagulation.	7.83
70	" "	7.70
142	Hemolysis. Very fluid and black. Bile-like odor.	4.53

Blood was removed from the ice box at this point and allowed to stand in the laboratory. It clotted about 5 hours later.

*Rabbit Blood Mixed Immediately with Citrate.*—To about 10 cc. of rabbit blood were added 100 mg. of dry sodium citrate; this was thoroughly shaken and kept in the ice chest.

Time after addition of citrate.	Remarks.	Amount of citrate per 1 cc. of blood.
hrs.		mg.
Immediately.		7.04
71	No coagulation.	7.29
95	Blood dark. No hemolysis. No coagulation.	6.05
117	Slight hemolysis.	4.30
141	Complete hemolysis.	Very faint test. Weight of precipitate 1.02mg. per 1.33 of blood.
191	Black, clotted.	

It will be seen from the above data that our results are in harmony with those of Battelli and Stern. The rapid disappearance of citrate from the circulation must be brought about, therefore,

by increased elimination or destruction by the tissues other than the blood. These conclusions were tested in the following experiments.

#### VI. The Elimination of Citrate.

Battelli and Stern (11) have shown that isolated tissues of different animals destroyed citrate *in vitro*. Its oxidation in the body, however, has never before been made the subject of investigation. Our experiments were carried out on rabbits, cats, and one dog. Sodium citrate was given subcutaneously in most of the experiments, but in some it was given intravenously or by mouth.

When 1 gm. of citrate per kilo was injected subcutaneously into rabbits receiving a diet of oats, appreciable amounts were found in the urine of the following 24 hours. A positive test for citrate was also obtained by means of the pentabromoacetate method. Citrate injected subcutaneously into cats was isolated from the urine. As shown in Table I, 4 to 8 per cent citrate were recovered in the urine of seven animals, 10 to 14 per cent in four, and 25.3 per cent and 28 per cent respectively in two others. The average amount of citrate in the thirteen experiments was 12 per cent, which was eliminated within 24 hours. The effect of hemorrhage was also studied in these animals. 22 to 56 gm. of blood drawn from rabbits weighing about 1.6 kilos failed to influence the elimination of citrate. The average amount eliminated was 12 per cent, the range of variation being 11.1 to 15.6 per cent. Oxidation is therefore not affected by hemorrhage, thus corroborating the results of our experiments on the oxidation of citrates by the blood *in vitro*.

Similar results were obtained in experiments in which sodium citrate was injected intravenously. When approximately 0.5 gm. per kilo was given in two experiments, 4 per cent in one and 13 per cent in the other were recovered from the urine at the end of 24 hours. In two other experiments in which 350 mg. of citrate per kilo were given, the amounts recovered from the urine were 1.5 and 2 per cent, but tests for citrate were negative after doses of 100 mg. per kilo.

It may be remarked that the citrate had to be given in divided doses since comparatively small amounts injected intravenously



produced marked symptoms and may cause death if the rate of injection is rapid. Dyspnea, convulsions, fibrillary twitching of the voluntary muscles and dilatation of the pupil were observed after the intravenous administration of 70 mg. of sodium citrate per kilo. When it was given by mouth minute amounts only could be detected in the urine though 3 gm. per kilo were administered.

The elimination of citrate in cats varied much more than in the rabbit. It was absent from the urine in one experiment, while the amount in nine others ranged from 5 to 58 per cent. As will be seen in Table III, in six of these experiments the amounts recovered varied approximately between 38 and 58 per cent. The average amount of citrate in the ten experiments was 30 per cent. That other carnivora probably react similarly to cats was suggested by results obtained in one dog in which 42 per cent of citrate was recovered at the end of 24 hours.

The presence of citrate in the urine, indicating incomplete oxidation in the body, would imply that the conditions under which

TABLE I.\*  
*The Elimination of Sodium Citrate in Rabbits.\*\**

Rabbit No. †	Weight.	Total amount of citrate injected.		Total eliminated.		Remarks.
		kg.	mg.	mg.	per cent.	
8	2.1	2,100		175.0	8.3	Total elimination in 24 hrs.†
9	1.58	1,600		153.0	9.05	" " " 24 "
10	2.00	2,000		136.0	6.80	" " " 24 "
11	1.67	1,700		144.0	8.40	" " " 24 "
12	1.325	1,350	1st.	144.0	10.7	" " " 24 "
	1.495	1,500	2nd.	206.0	13.7	Died 2 days after injection.
13	2.15	2,200		616.0	28.0	" soon after 2nd "
14	1.955	1,950		273.0	14.0	Total elimination in 24 hrs.
15	1.800	1,800	1st.	84.4	4.7	" " " 24 "
	1.755	1,750	2nd.	71.0	4.1	" " " 24 "
	1.765	1,750	3rd.	142.0	8.1	" " " 24 "
16	1.585	1,600		192.5	12.0	" " " 24 "
17	1.49	1,500		380.0	25.3	" " " 24 "

\* No citrates found in urines prior to citrate injection.

\*\* 1 gm. of anhydrous sodium citrate per kilo injected subcutaneously.

† Previous diet, oats.

‡ None or negligible amounts found later.

this process takes place are probably different in the isolated tissue and in the living organism. Battelli and Stern have demonstrated that the salt may be rapidly decomposed outside the body when brought in contact with extracts of animal organs. Although the factors involved in either case are as yet largely un-

TABLE II.

*Experiments on the Influence of Hemorrhage on the Elimination of Sodium Citrate in Rabbits.\*†*

Rabbit No.	Weight.	Total amount of citrate injected.	Total eliminated.		Remarks.
			mg.	per cent	
18	1.76	1,700	223.0	13.0	Symptoms doubtful. 35 gm. of blood obtained in 24 hrs. and 21 gm. more 1 hr. and 20 min. before injection of citrate (ear vein).
19	2.03	2,000	228.0	11.4	No symptoms. 42 gm. of blood drawn 19 hrs. before injection of citrate. 12 gm. more about 1 hr. before injection (ear vein).
9	1.61	1,600	165.0	10.3	No symptoms. 31 gm. of blood drawn 20 hrs. before injection of citrate. 24 gm. 1 hr. before injection (ear vein).
20	2.15	2,200	343.0	15.6	Tremors and muscular incoordination noticed in 30 min., which disappeared in 2½ hrs. 23 cc. of blood removed from heart.
21	2.19	2,200	245.0	11.1	Muscular incoordination, dyspnea, salivation, and convulsions appeared in 55 min. 22 cc. of blood removed from heart. Rabbit dead next day. Blood clot found in pericardial sac.

\* Had previously received citrate.

† 1 gm. of anhydrous sodium citrate per kilo injected subcutaneously. Elimination complete in 24 hrs.

known, some data on the subject obtained by those investigators are suggestive. They reported that with the method they employed sodium chloride, or phosphate, when present in sufficient concentration, as well as bile, arsenious acid, salicylic acid, and aldehyde, retard the oxidation of citrate. The presence of sub-

TABLE III.

*The Elimination of Sodium Citrate in Cats.\**

Cat No.	Weight.	Total amount of citrate injected.		Total eliminated.		Remarks.
		kg.	mg.	mg.	per cent	
1	1.79	1,800	97.5	5.6		No symptoms. Eliminated in 48 hrs.
2	1.46	1,450	223.5	15.4		" " " " 24 "
3	0.95	950	363.0	38.2		" " " " 24 "
4	0.93	950	None.	0.0		" " " " 24 "
5	2.23	2,200	845.0	38.5		Symptoms appeared in 1½ hrs. Marked muscular incoordination, dilated pupils, twitching of muscles of neck; difficult deglutition. Cat looked normal after 5 hrs. Eliminated in 24 hrs.
6	2.66	2,700	1,256.0	46.5		Depression, only symptom, appeared in 1 hr., lasted a few hrs. Eliminated in 24 hrs.
7	1.93	1,950	829.0	42.5		Symptoms appeared in ½ hr. Muscular incoordination, marked twitching of ears and neck, depression; continued 3 days. Eliminated in 24 hrs.
8	3.26	3,300				Marked muscular incoordination, dyspnea, coma, convulsions, appeared in ¾ hr. Died before end of first 24 hr. period.
9	1.75	1,800	259.0	14.3		Convulsions, muscular twitching, dilated pupils, increased reflexes, appeared in ¾ hr.; persisted 2 days but subsided on 3rd day. Eliminated in 24 hrs.
10	2.05	2,000	917.0	45.9		Dyspnea and tremors appeared in ½ hr. and subsided within a few hrs. Eliminated in 48 hrs.
11	2.45	2,500	1,467.0	58.7		Muscular incoordination, slight convulsions, and tremors in 1½ hrs. Symptoms still present next day but were mild. Nearly all eliminated in 48 hrs.

\* 1 gm. of anhydrous sodium citrate per kilo injected subcutaneously. Elimination complete in 24 hrs.

stances in the fluids and tissues in the body exerting an inhibitory effect in a similar manner would account, therefore, for the difference in the rate of oxidation which seems to exist when citrate is acted upon in the organism and *in vitro*. This is difficult to explain, however, in view of their observations that the rate of oxidation of sodium citrate is greatest in fresh tissues, the process becoming progressively slower with age, but the large number of factors involved may explain this apparent contradiction. Battelli and Stern found that the speed of oxidation varies directly with the amount of oxygen but is retarded by acid, the concentration of citrate also playing an important part. Hence the retarding influence of the age of the tissues on the oxidation of citrate apparently holds good, provided all other conditions are the same, and does not apply to (and in no wise affects) the comparison of the results obtained when the substance is administered to animals.

The greater amounts of sodium citrate eliminated in the urine of carnivorous animals than in that of the rabbit may be due to a difference in the rate of oxidation in these animals or to diuresis. The latter possibility may be excluded, however, as it was noticed that the kidney was stimulated to greater activity in most cases in the rabbit after the salt was given, while the volume of urine in cats was not affected by this treatment.

### *VII. Observations on the Toxicity of Citrate.*

When sodium citrate was administered in sufficient amounts, the symptoms observed indicated disturbance of the central nervous system, muscles, respiratory organs, and intestinal tract. The effective dose varied considerably with the mode of administration. 3 gm. per kilo given by mouth usually failed to cause nervous or muscular symptoms, but in some cases this amount caused violent purgation and death. A dose of 5 gm. per kilo caused paralysis of the extremities and death. The duration of life was usually about 4 hours, but death occurred in one experiment within 18 minutes.

In experiments in which it was given subcutaneously much smaller doses were required to produce toxic action. After 2 gm. per kilo were introduced, severe symptoms were observed within

TABLE IV.

*The Toxicity of Sodium Citrate in Rabbits. Administration by Mouth.*

Rabbit No.	Weight.	Dose per kilo.	Symptoms.	Duration of life.
	gm.	gm.		
22*	1,315	3.0	Twitching of muscles in 1 hr. 45 min.	Survived.
23*	1,350	3.0	Violent diarrhea in 2 hrs. 40 min.	Chloroformed.
24**	2,670	3.0	Diarrhea.	Dead next day.
25*	2,285	3.0	"	Survived.
26**	2,060	3.0	"	"
27*	2,285	3.0	"	"
6**	1,350	5.0	Paresis of extremities in 2½ hrs.	4½ hrs.
5*	1,280	3.0	Tremors. Peristalsis stimulated after administration of citrate.	
28*	1,415	3.0	Diarrhea in 2 hrs.	
29*	1,900	5.0	Slight symptoms in 35 min.	4 "
30**	1,950	5.0	Violent convulsions in 15 min.	18 min.
31**	1,860	5.0	Slight symptoms noted in 20 min., coma and paralysis in 4 hrs.	4 hrs.
32*	1,380	5.0	Paresis of hind legs in 2 hrs. 25 min.	4 "

\* 10 per cent administered.

\*\* 20 " " "

10 to 20 minutes and death in 1 to a few hours. In a large proportion of the experiments in which a dose of 1 gm. of the salt per kilo was given no manifestation of any untoward effects occurred. Mild symptoms developed in a few cases, while evidence of severe intoxication, terminating in recovery soon after (Rabbit 43), was observed in one case only. On the other hand, 1.5 gm. of sodium citrate per kilo were injected subcutaneously without causing any signs of disturbance and the rabbits survived. It might be added in this connection that a dose of 1 gm. of the salt per kilo, given subcutaneously to rabbits, likewise failed to cause any symptoms even when considerable amounts of blood were withdrawn previous to the administration of citrate.

The toxic effects were more pronounced in cats (Table III). 1 gm. per kilo given subcutaneously to eleven cats caused depres-

sion, muscular incoordination, dyspnea, twitching of muscles, convulsions, dilatation of pupils, coma, and increased reflexes. One cat (No. 8) was found dead the morning after injection. Only one experiment was carried out on a dog, which received subcutaneously 1 gm. per kilo, but no symptoms were noticed.

TABLE V.

*The Toxicity of Sodium Citrate in Rabbits. Subcutaneous Injection.*

Rabbit No.	Weight.	Dose per kilo.	Remarks.
	gm.	gm.	
33	1,845	1.0	Pregnant rabbit. Rapid respiration, restlessness, fright, in 10 min. Survived.
34	1,405	1.0	Looked frightened; no symptoms; survived.
1*	2,040	0.5	Tremors appeared in 10 min. 15 cc. of blood taken 5 min. after citrate was given.
2	1,945	1.0	Symptoms appeared 25 min. after injection of citrate.
35	2,250	2.0	Restlessness, forced movements, convulsions, muscular twitching, appeared in 20 min.; lived less than 18 hrs.
36	1,999	2.0	No symptoms.
37	1,505	1.5	" "
38	1,720	2.0	Symptoms appeared in 10 min. Lived 1 hr. 10 min.
39	1,500	1.5	No symptoms.
40	1,880	1.5	" "
41	1,135	1.0	No marked symptoms; survived.
42	650	1.0	Slight muscular incoordination observed in 1 hr. 15 min. Survived.
43	1,215	1.0	Muscular twitching, incoordination, convulsions, appeared in 1 hr. 10 min. Lived 17 days.

\* Urethane anesthesia.

Observations were also made on the action of sodium citrate when injected intravenously. Symptoms were present after small doses of the salt were introduced rapidly. Thus a dose of 67 mg. of sodium citrate per kilo injected into the ear vein of a rabbit in 85 seconds was followed by slight muscular twitching. The symptoms were more marked in another case after a dose of 86 mg. of citrate per kilo was injected in 2 minutes. Convulsions were noticed in a third rabbit which received 90 mg. of citrate in  $4\frac{1}{2}$

minutes. Severe intoxication was produced by 115 mg. of citrate per kilo injected in 1 minute, and 147 mg. per kilo, introduced at the rate of about 23 mg. per minute. Larger doses may be injected, however, without fatal termination if the injections are given in divided doses at sufficiently long intervals. Somewhat more than 0.33 gm. per kilo was introduced in 65 minutes into two rabbits without causing death. In a third experiment 0.46 gm. per kilo, injected in 2 hours and 40 minutes, did not produce death, while another rabbit survived 0.7 gm. of sodium citrate per kilo, injected in 74 minutes. It is worthy of remark that no after effects occurred in any of these rabbits. That the toxicity of citrate depends largely upon the speed of injection was shown in the two following experiments.

In one case in which a solution of 2.5 per cent citrate was introduced at the rate of about 1 cc. per minute with an occasional interval of a few minutes' rest, a dose of 1.11 gm. per kilo proved fatal. In another case the fatal dose was 1.66 gm. per kilo, the time of injection being extended over a period of  $3\frac{1}{2}$  hours. The rate of injection in this case varied between 0.67 and 1.5 cc. per minute except towards the end of the experiment when the speed was increased to 4 cc. per minute.

*Rabbit 44.—Female, white.*

Aug. 2, 1916. Weight 1,940 gm.

Aug. 3. Ether and ethyl chloride anesthesia. Cannula in jugular vein. 10.54 a.m. Injection of 2.5 per cent citrate begun. 1 cc. injected in 1 minute. 11.04 a.m. 9 cc. injected, 1 cc. per minute. 11.10 a.m. 10 cc. injected, 1.5 cc. per minute. Total injection, 20 cc. 11.11 a.m. Dyspnea after total of 21.5 cc. 11.19 a.m. Injection of 10 cc., 1.25 cc. per minute. 11.28 a.m. Injection of 10 cc., 1 cc. per minute. Total injection, 40 cc. 11.20 a.m. Periodic suspension of respiration. 11.27 a.m. Spasms of muscles of fore legs. Dyspnea very marked. 11.30 a.m. Injection of 2.5 cc. Total injection 42.5 cc. General twitching of muscles. Convulsions; fibrillary contractions. 11.31 a.m. Respiration stopped for a few seconds. 11.33 a.m. Injection of 2.5 cc. Total injection, 45 cc. Convulsions 0.5 minute later. 11.34 a.m. Injection of 2 cc. Total injection, 47 cc. Injections stopped. 11.35 a.m. Cheyne-Stokes respiration. 11.37½ a.m. Injection resumed. 11.45½ a.m. Injection of 5 cc., 1.5 cc. per minute. Total injection, 52 cc. 12 m. Injection of 10 cc., 0.66 cc. per minute. Total injection, 62 cc. 12.15 p.m. Injection of 14 cc. Total injection, 76 cc. 12.12 p.m. Respiration stopped for a few seconds. 12.13½ p.m. General spasms; general fibrillary contractions. 12.15 p.m.

Convulsions. 12.29 p.m. Injection of 16 cc., 1 cc. per minute. Total injection, 92 cc. Respiration stopped for a few seconds. 12.52 p.m. Respiration stopped for a few seconds, then slight convulsions. Injection stopped. 1.47 p.m. Injection resumed. (5 minute interval.) 1.50 p.m. Injection of 11 cc., 4 cc. per minute. Muscular tremors observed but not marked. Total injection, 103 cc. 1.54 p.m. Injection of 8 cc., 2 cc. per minute. Total injection, 111 cc. 1.52½ p.m. Tremors, convulsions. 1.54 p.m. Convulsions continued. No respiration for 1 minute. 1.55 p.m. Respiration very feeble and infrequent. Convulsions. 2.19 p.m. Injections resumed after 25 minute interval. 2.26 p.m. Injection of 15 cc., 2 cc. per minute. Total injection, 126 cc. 2.27 p.m. No respiration. 2.27½ p.m. Injection of 3 cc. Total injection, 129 cc. 2.28 p.m. Animal dead. 2.30 p.m. Upon opening the thoracic cavity the heart was found to be beating.

*Rabbit 45.*—Female, white.

Aug. 2, 1916. Weight 1,100 gm.

Aug. 3. Ether anesthesia; injected into jugular vein by means of a cannula. 2.58 p.m. Injection of 2.5 per cent citrate begun. 3.03 p.m. Injection of 10 cc. 3.12 p.m. Injection of 2 cc. Dyspnea, struggled. 3.14 p.m. Injection of 4 cc. Cheyne-Stokes respiration. Marked twitching of muscle. 3.16 p.m. 5 cc. injected. Tetanus, respiration stopped. 3.17 p.m. 1 cc. injected. Respiration returned but stopped. 3.18 p.m. to 3.19 p.m. A few respirations and then stopped. 3.20 p.m. The same but stronger and deeper, and lasted longer. 3.24 p.m. Respiration much improved. Cheyne-Stokes still present but apnea of short duration. 3.25 p.m. Injections resumed. 3.30 p.m. Struggled. 3 cc. injected. 3.34 p.m. 3 cc. injected. Tonic rigidity of posterior extremities. 3.36 p.m. 2 cc. injected. Convulsions, dyspnea. 3.40 p.m. 4 cc. injected. General fibrillary twitching. Respiration stopped. 3.42 p.m. Respiration returned but stertorous and soon stopped. 3.50 p.m. 7 cc. injected. Cheyne-Stokes respiration. Salivation very marked. 3.56 p.m. 7 cc. injected. Convulsions. Respiration stopped a few seconds and then returned but was slow and labored. 4 p.m. Total injection, 49 cc. Respiration stopped. Heart paralyzed.

*Autopsy.*—Performed at 4.03 p.m. Heart injected and distended with blood; paralyzed.

No peristalsis noticed after injection of citrate in this rabbit or in Rabbit 44.

In experiments on dogs in which 2.5 per cent sodium citrate was given intravenously, 100 mg. per kilo, injected at the rate of 50 to 70 mg. per kilo per minute, stoppage of respiration, and sometimes also arrest of the heart were noted. The fatal dose, however, is much larger (Dog 4) as 0.5 gm. per kilo given in four doses, administered in 64 minutes, did not cause death. In another experiment, 0.375 gm. per kilo, given in 38 minutes in di-



TABLE VI.

*The Toxicity of Sodium Citrate in Rabbits. Intravenous Injection.*

Rabbit No.	Weight.	Dose per kilo.*	Period of injection.	Remarks.
	gm.	gm.	min.	
46	1,715	0.147	8	Injections at irregular intervals; dyspnea after 75 mg. per kilo were injected; 2 min. after the first injection (147 mg. per kilo) tremors and convulsions. Total injection 0.6 gm. per kilo in 3 hrs. Survived.
47	2,005	0.193	4½	Injected 250 mg. in 2 min. 4 min. later 137 cc. in 2½ min.; convulsions, dyspnea. Total injection 0.46 gm. per kilo in 2 hrs. Survived. Subcutaneous injection 8 days later (Table V).
1	1,965	0.115	1	Convulsions; dilated pupils; muscular twitching after 115 mg. per kilo. Total injection 0.35 gm. per kilo in 65 min. Survived.
48	2,775	0.072	4½	Total injection 0.2 gm. per kilo in 72 min. Survived.
49	2,470	0.086	2	Dyspnea and convulsions at end of first injection. Total injection 0.25 gm. in 5 min. Survived.
50	1,930	0.070	1½	Muscular twitching after first injection. Total injection 0.354 gm. per kilo in 65 min. Survived.
51	1,670	0.090	4½	Convulsions; muscular twitchings after injection of 90 mg. per kilo. 0.7 gm. per kilo injected in 74 min. Survived.
44	1,940	0.225	17	Dyspnea after 0.225 gm. per kilo; paralysis of respiration after 1.66 gm. per kilo in 3 hrs. 34 min.; heart still beating when thorax was opened.
45	1,100	0.272	14	Dyspnea and struggling after 272 mg. per kilo were injected. Total injection, 1.1 gm. per kilo in 62 min.
52	1,700	0.160	8½	Violent convulsions after 160 mg. per kilo; died after injection of 0.428 gm. per kilo in 19.5 min.

\* First injection.

vided doses proved fatal, the last dose of 175 mg. per kilo being injected at the rate of 50 mg. per kilo per minute. In a third experiment, the fatal dose (Dog 2) was 0.9 gm. per kilo, given in divided doses in a period of 1 hour and 47 minutes.

TABLE VII.

*The Toxicity of Sodium Citrate in Dogs. Intravenous Injection.*

Dog No	Weight	Dose per kilo *	Period of injection *	Total amount per kilo	Total time	Remarks
	kg	gm	min	gm	min	
1	6 9	0 100	2	0 326	33	Last injection 174 mg. per kilo. Cardiac and respiratory paralysis. No symptoms noticed before
2	6 5	0 100		0 9	107	Respiration ceased after first injection but returned in 1 min.; heart stopped after last injection of 950 mg.
3	5 3	0.100	1½	0 31	52	Respiration stopped after first injection; last injection 100 mg. per kilo; paralysis of respiration.
4	4 32	0 090	½	0 5	64	Total dose not fatal.
5	5 1	0 100	1½	0 2	17	Respiration stopped and heart beat faintly after first injection.

\* First injection.

*VIII. Experiments on Cumulation and Tolerance.*

The action of citrate considered thus far was confined mainly to single doses except in the case of the experiments in which intravenous injections were made when small doses were given at brief intervals. Attention was therefore directed to the behavior of the salt when given at intervals of 1 or more days. Amounts that failed to produce any symptoms when given the first time, were very toxic, sometimes causing death, when repeated within 1 to 3 days. Cumulative action was observed after subcutaneous injections as well as when the salt was given by mouth, as shown in the experiments given below. Robertson and Burnett (7) reported similar experiments. They found, on the contrary, that tolerance may be induced by the subcutaneous injections of sodium citrate to rabbits. Their experiments were carried out with gradually increasing doses, extending over a much longer period than ours, which may account for the different results obtained.

*Rabbit 33.*—Female, white.

Apr. 7, 1915. Received in the laboratory and placed on a diet of oats and hay. Weight 1,845 gm. (Pregnant.)

June 8, 3.45 p.m. Subcutaneous injection of 16 cc. of 10 per cent sodium citrate (1.0 gm. per kilo). 3.55 p.m. Respiration rapid. Restless. Frightened. Ears erect. 4.35 p.m. No symptoms.

June 9, 3.30 p.m. Subcutaneous injection of 20 cc. of sodium citrate. (1.0 gm. per kilo.) 3.45 p.m. Dyspnea. Muscular tremors. Salivation. 3.55 p.m. Convulsions. Dyspnea. Both marked and lasted about 1 minute. Rabbit improved. 4 p.m. Appearance almost normal. Pupils much dilated.

June 10. Weight 1,635 gm. 11.55 a.m. Subcutaneous injection of 20 cc. of sodium citrate (1.25 per kilo). No urination but defecation. 12.06 p.m. Dyspnea. Convulsions. Muscular twitching. Fibrillar contraction. 1.30 p.m. Symptoms continued.

June 11. Loss of appetite. Lay in cage in one position all day.

June 12, 9 a.m. Animal dead.

*Rabbit 34.*—Female, Belgian.

May 20, 1915. Received in the laboratory and placed on a diet of oats and hay. Weight 1,010 gm.

June 8. Weight 1,405 gm. 3.47 p.m. Subcutaneous injection of 14 cc. of 10 per cent sodium citrate (1.0 gm. per kilo). 3.55 p.m. Frightened. Ears erect. 4.35 p.m. No symptoms.

June 9, 3.31 p.m. Subcutaneous injection of 20 cc. of 10 per cent sodium citrate (1.5 gm. per kilo). 4 p.m. Shook head occasionally but no other symptoms. 4.05 p.m. Muscular tremors.

June 10, 11.58 a.m. Weight 1,460 gm. Subcutaneous injection of 20 cc. of 10 per cent sodium citrate. Almost immediate urination and defecation.

June 14. Averaged 50 gm. of oats a day since last injection. Weight 1,375 gm. 2.01 p.m. Subcutaneous injection of 21 cc. of 10 per cent sodium citrate (1.5 gm. per kilo). 2.10 p.m. Passed hard feces. 2.45 p.m. Posterior extremities rigid. Rigidity more marked in handling. Tonic rigidity of posterior extremities. Salivation. Fibrillary twitchings of muscles. 2.47 p.m. Convulsions. 4.15 p.m. Temperature 110° F. 4.40 p.m. Animal dead.

*Rabbit 39.*—Female, maltese.

May 20, 1915. Received in the laboratory and placed on a diet of oats and hay. Weight 1,370 gm.

June 11. Weight, 1,735 gm. 3.34 p.m. Received subcutaneous injection of 25 cc. of 10 per cent sodium citrate solution (1.5 gm. per kilo). 4.25 p.m. Respiration accelerated. No other symptoms.

June 14. Weight 1,555 gm. Ate about 25 gm. of oats in 2 days. 2 p.m. Received subcutaneous injection of 25 cc. of 10 per cent sodium citrate solution (1.6 gm. per kilo). 2.16 p.m. Paresis of anterior extremities. Pupils normal. 2.46 p.m. Marked convulsions of posterior parts. Salivation. 4.15 p.m. Temperature 108° F.

June 15, 9 a.m. Animal dead. No autopsy.

## DISCUSSION.

Since it has been assumed by a number of investigators that the action of tartrate, citrate, and oxalate is due to the same mechanism, namely, to calcium precipitation, a comparison of their behavior in the body may prove of value. Their fate when administered to animals has received some attention at the hands of several observers, as the following brief résumé of the literature will show.

It has been shown by Hildebrandt (12) and corroborated by Dakin (13) that nearly all of the oxalate may be oxidized after its subcutaneous injection into rabbits, while Faust (14), who in the same way administered the acid to dogs, recovered from the urine almost the entire amount given.

The behavior of tartrates in this respect is less satisfactory, as the results obtained by several investigators in different animals are not concordant. According to Pohl (15) when tartrates are given to dogs and rabbits, 33 to 50 per cent of the amounts introduced were eliminated unchanged in the urine of the animals. Underhill, Wells, and Goldschmidt (16) observed only inappreciable quantities of tartrate in the urine of rabbits that had received subcutaneously moderately large doses of the salts. The results of our experiments with citrates show, therefore, that this salt is probably decomposed in the body of carnivorous animals more readily than the oxalate or tartrate.

The toxic action of citrate, tartrate, and oxalate indicates that they also differ in this respect. For the present, however, our discussion will be confined to the toxicity of citrate and tartrate, reserving a comparison of the action of oxalate and its allied compounds for a forthcoming communication.

When citrate was given intravenously, subcutaneously, or by mouth no after effects were observed. Neither sugar nor albumin was present in the urine which was examined for several days after toxic doses of citrate were administered. Symptoms developed rapidly, especially after large doses, and ended in recovery or death within a few hours. In experiments on cats symptoms persisted for 1 or even 2 days, but this occurred so rarely that it may be regarded as exceptional. The action of citrate is therefore acute.

The action of the tartrate when given subcutaneously was almost invariably subacute and caused a marked nephritis in the rabbit and in cats. As observed by Salant and Smith (17) and by Karsner and Denis (18), somewhat larger doses of the salt were required to produce renal injury in the cat.

The two salts also differed in experiments in which they were given by intravenous injections, the dose of tartrate required to produce acute symptoms being much larger than that of citrate. Thus, acute toxic effects were first observed when 1.5 gm. per kilo were introduced at the rate of 100 to 200 mg. per kilo per minute. The dose required to produce the effect was even larger when the rate of injection was decreased, while the minimum fatal dose of tartrate when it was introduced directly into the blood stream was about 4.2 gm. per kilo. The divergence in the behavior of the two salts in the body was especially marked with small doses when injected intravenously. About 0.4 gm. of sodium tartrate per kilo caused renal injury in the rabbit, nervous or muscular symptoms after such a dose being rare. The same amount of citrate per kilo was found to be highly toxic and may cause death. When given by mouth the surely fatal dose in rabbits was about the same for tartrates and citrates, but there was a notable difference in the effect of the two salts when smaller doses were used. We have found that about 3 gm. of sodium citrate per kilo were just sufficient to produce purgation and may be fatal in some cases. Underhill, Wells, and Goldschmidt (19) observed renal changes in rabbits which had received by mouth 3 to 4 gm. of rochelle salt per kilo.

The behavior of citrate and tartrate when repeated doses were given also showed that their action is not the same. When gradually increasing amounts of the latter were injected subcutaneously into rabbits a tendency to tolerance developed, while the opposite, as may be recalled, was observed with citrates.

It is evident, therefore, from the data presented above that the acute effect of citrate is considerably greater than that of tartrate, which has also been shown by Salant and Hecht (9) in experiments upon the isolated heart. That the tartrate is nevertheless more poisonous than the citrate may be accounted for by the rate of oxidation of the two salts, the latter probably being more readily destroyed in the body than the former, which would

appear from the results of Pohl cited above. The action of these salts is thus similar to ethyl and methyl alcohol. It had been shown that the acute toxic dose of methyl alcohol is larger than that of ethyl alcohol, the oxidation of the former being slower and incomplete. According to Pohl methyl alcohol is oxidized to formic acid and formaldehyde, which are more poisonous than the original substance. Perhaps tartrate undergoes a similar transformation in the body, the products of decomposition being more toxic than the salt from which they are derived.

#### SUMMARY AND CONCLUSIONS.

1. Sodium citrate disappears rapidly from the circulation after intravenous injection into cats and dogs, but this is retarded when the doses are repeated. Oxidation and elimination are probably much slower when large doses are given at frequent intervals.

2. The rate of oxidation of citrate is considerably greater in rabbits than in cats. The amount of citrate eliminated in the urine after subcutaneous injection averaged 12 per cent in the former and about 30 per cent in the latter.

3. Hemorrhage in rabbits does not affect the elimination of citrate, which is not destroyed when added to blood *in vitro*.

4. Large doses of sodium citrate when given by mouth render the urine alkaline. Only negligible quantities are sometimes found in the urine and in the blood after feeding sodium citrate.

5. The toxicity of sodium citrate when given intravenously depends upon the rate of injection, the fatal dose varying between 0.4 and 1.6 gm. per kilo (approximately), but about 70 mg. may produce symptoms.

6. Only large amounts of citrate are toxic when ingested.

7. Large doses given subcutaneously showed cumulative action.

8. No citrate was found in the urine when doses under 0.5 gm. per kilo were given intravenously.

9. The toxicity of sodium citrate depends upon the rate of its oxidation in the body, being more toxic for animals in which larger quantities are eliminated unchanged.

10. Sodium citrate mixed with blood in the proportion of 1:100 inhibited coagulation for 4 days when kept at a temperature of 10° C.

11. Acute action only was observed in experiments with sodium citrate, thus differing from tartrate, the action of which may be acute or subacute.

12. The acute effect of tartrate is less than that of citrate.

13. A method for the quantitative determination of small amounts of citric acid in urine, based on Denigès' reaction has been described. A modification of this method served for the approximate estimation of very small amounts of citrate in blood.

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## KAFIRIN, AN ALCOHOL-SOLUBLE PROTEIN FROM KAFIR, ANDROPOGON SORGHUM.\*

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Kafir has become an important crop in the United States during the last decade. In 1910 three million acres were under cultivation and the value of the crop was thirty million dollars. Hitherto, no study has been reported on the proteins of kafir. It has been known for some time that this cereal contains an alcohol-soluble protein. Osborne,<sup>1</sup> among others, states that he found it to contain such a protein, but he did not make a study of it. The seeds used in our experiments were grown in Kansas in 1915 and were of the variety known as dwarf kafir. These contained a strong red coloring substance soluble in alcohol, which accounts for the color of the alcoholic extracts described below.

Nitrogen determinations on the kafir meal showed that it contained 11.7 per cent of protein ( $N \times 6.25$ ). Of this, boiling 60 per cent alcohol extracted 7.9 per cent of protein, based on the nitrogen content of the extract. We were able to isolate 5.2 per cent of pure protein, dried at 110°C., by the use of alcohol ranging from 60 to 70 per cent by volume. As no account was taken of the losses occurring in the various manipulations during the preparation of the protein, this yield indicates that most of the nitrogen extracted was in the form of protein.

Since this is the first protein isolated from kafir and constitutes the greater part of the protein contained therein, we have named it *kafirin*.

\* A preliminary paper was presented at the Urbana meeting of the American Chemical Society (*Science*, 1916, xliv, 217).

The kafir used in these experiments was obtained from the Office of Cereal Investigations, Bureau of Plant Industry, United States Department of Agriculture. It is known as Dwarf Blackhull kafir and is described by Ball, C. R., and Rothgeb, C., *U. S. Dept. Agric., Farmers' Bull.* 552, 1913, p. 8.

<sup>1</sup> Osborne, T. B., *The Vegetable Proteins*, London, 1909, 80.



In its ultimate composition kafirin closely resembles zein from maize, as is shown by a comparison of the following values.

	Kafirin.	Zein.*
C. ....	55.19	55.23
H. ....	7.36	7.26
N. ....	16.44	16.13
S. ....	0.60	0.60
O. ....	20.41	20.78

\* Chittenden, R. H., and Osborne, T. B., *Am. Chem. J.*, 1891, xiii, 453; 1892, xiv, 20.

While zein is very soluble in 70 per cent alcohol at all temperatures, kafirin requires a large quantity of the same strength of alcohol to effect solution. Kafirin is much more soluble in hot alcohol than in cold and even rather dilute solutions will form a jelly on cooling. On this account it was necessary to use the large volumes of alcohol stated in the experimental part of this paper, and to filter the extracts while hot. Kafirin also coagulates easily, while an alcoholic solution of zein does not coagulate when heated.

A striking difference between kafirin and zein is found in the distribution of the nitrogen in these proteins as shown by the following figures.

N	Kafirin.	Zein.*
Humin .....	0.17	0.16
Amide .....	3.46	2.97
Basic .....	1.04	0.49
Non-basic .....	11.97	12.51
Total .....	16.64	16.13

\* Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, 1903, xxv, 323.

Apparently a further difference exists between kafirin and zein in the proportion of the diamino-acids which these proteins yield. The following figures give the percentages of these acids as shown by an analysis of kafirin by the Van Slyke method and an analysis of zein made by Osborne and Jones<sup>2</sup> by Kossel's direct method.

<sup>2</sup> Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*, 1910, xxvi, 227.

In the analysis of kafirin 0.78 per cent of cystine was precipitated with the phosphotungstates of the other bases and a corresponding correction has been made in the figures.

	Kafirin.	Zein.
Arginine.....	1.58	1.55
Lysine.....	0.90	0.00
Histidine.....	1.00	0.82
Tryptophane.....	Present.	0.00

It is thus seen that kafirin contains lysine and tryptophane, both of which are lacking in zein, and which are necessary for animal nutrition.

Further investigations on the proteins of kafir are in progress.

#### EXPERIMENTAL.

*Preparation of Kafir Meal.*—The kafir seeds were ground to a meal. This was placed in stoppered bottles and used for the experiments described below.

*Preparation of Kafirin.*—The kafir meal was stirred into eight to ten parts of boiling 70 per cent ethyl alcohol (by volume). This mixture was heated for an hour in a double boiler made by placing a pail containing the extraction mixture in a somewhat larger pail containing water heated to about 80°C. To avoid coagulation of the protein, the mixture must be frequently stirred to prevent the meal from settling as a hard cake on the bottom of the pail and becoming overheated. If none of the kafirin had been coagulated the hot mixture was readily filtered on a Buchner funnel and the deep red filtrate again filtered through a folded filter. In this way a perfectly clear liquid was obtained. This was concentrated under diminished pressure to about one-half its original volume and poured into a large quantity of chilled, distilled water.

The kafirin first appeared as a milky suspension which showed no tendency to flock. The addition of sufficient sodium chloride solution while stirring caused the protein to settle as a bulky flocculent or granular precipitate, leaving the supernatant liquid almost clear. This precipitate was washed several times by decan-

tation and then filtered off on cheese-cloth, and the water held in the precipitate was pressed out as far as possible by wringing the cloth. The protein was disintegrated and suspended in absolute alcohol for 24 hours. The alcohol assumed a deep red color from the coloring substances carried down by the protein. To remove all the color it was necessary to renew the absolute alcohol two or three times. The kafirin was then filtered off and suspended in absolute ether for 24 hours. The ether was poured off and the kafirin squeezed out in a cloth and disintegrated in a meat chopper, which left it in a granular condition. It was then placed in a vacuum desiccator over sulfuric acid until freed from ether, and finally dried in the vacuum oven, the temperature being gradually raised to  $110^{\circ}\text{C}$ . The dried kafirin was then easily ground to a flour in a mill and obtained as a gray powder. Preparations 1 to 12, inclusive, were made in this way.

In order to determine whether the kafirin could be obtained in a purer state, Preparation 13, after precipitation with water and filtering, was redissolved in hot 70 per cent alcohol. A large volume of alcohol is necessary in redissolving the kafirin as coagulation occurs if too small a volume is used. The kafirin was reprecipitated by pouring into a large volume of water and was then treated as described above. Comparison of the analytical results makes it apparent that there is no advantage to be gained by purification in this manner, which at the same time greatly reduces the yield.

Analyses of Preparations 1 to 13, calculated on a moisture-free basis, gave the following results.

	Preparation 1.				Preparation 2.	
	I.	II.	Average.	Ash-free.		Ash-free.
C.....	55.22	55.01	55.12	55.20	55.36	55.52
H.....	7.31	7.25	7.28	7.29	7.29	7.31
N.....	16.40	16.47	16.43	16.46	16.20	16.25
S.....	0.63		0.63	0.63	0.55	0.56
O.....				20.42		20.36
Ash.....	0.17	0.17	0.17		0.26	

	Preparation 3.				Preparation 4.	
	I.	II.	Average.	Ash-free.		Ash-free.
C.....	55.07	55.35	55.21	55.38	55.07	55.21
H.....	7.20	7.25	7.22	7.26	7.24	7.26
N.....	16.30	16.15	16.22	16.28	16.37	16.41
S.....	0.71		0.71	0.71	0.66	0.66
O.....				20.37		20.46
Ash.....	0.33				0.28	

	Preparation 5.				Preparation 6.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	55.33	55.10	55.21	55.33	54.86	54.90	54.88	54.99
H.....	7.38	7.35	7.36	7.38	7.25	7.25	7.25	7.27
N.....	16.36	16.29	16.32	16.35	16.32	16.36	16.34	16.38
S.....	0.62		0.62	0.62	0.49	0.52	0.51	0.51
O.....				20.32				20.85
Ash.....	0.22				0.22			

	Preparation 7.				Preparation 8.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	55.38	55.42	55.40	55.60	55.02	54.88	54.95	55.07
H.....	7.35	7.32	7.34	7.36	7.47	7.44	7.45	7.47
N.....	16.31	16.46	16.38	16.43	16.41	16.55	16.48	16.53
S.....	0.56		0.56	0.56	0.64		0.64	0.64
O.....				20.05				20.29
Ash.....	0.31				0.20			

	Preparation 9.				Preparation 10.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	55.12	54.94	55.03	55.19	54.80	54.87	54.83	55.06
H.....	7.36	7.41	7.38	7.40	7.30	7.46	7.38	7.41
N.....	16.57	16.61	16.59	16.64	16.61	16.43	16.52	16.58
S.....	0.50		0.50	0.50	0.60		0.60	0.60
O.....				20.27				20.35
Ash.....	0.24				0.47			

	Preparation 11.				Preparation 12.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	54.98	54.81	54.89	55.03	54.79	54.75	54.77	54.89
H.....	7.22	7.34	7.28	7.30	7.37	7.51	7.44	7.46
N.....	16.48	16.33	16.40	16.45	16.43	16.39	16.41	16.45
S.....	0.59			0.59	0.60		0.60	0.60
O.....				20.63				20.60
Ash.....	0.23				0.21		0.21	

	Preparation 13 (redissolved).			
	I.	II.	Average.	Ash-free.
C.....	54.92	54.90	54.91	55.04
H.....	7.41	7.42	7.41	7.43
N.....	16.54	16.40	16.47	16.51
S.....	0.65		0.65	0.65
O.....				20.37
Ash.....	0.20		0.20	

*Summary of Analyses of Kafirin.*

Preparation No.	1.	2.	3.	4.	5.	6.	7.
C.....	55.20	55.52	55.38	55.21	55.33	54.99	55.60
H.....	7.29	7.31	7.26	7.26	7.38	7.27	7.36
N.....	16.46	16.25	16.28	16.41	16.35	16.38	16.43
S.....	0.63	0.56	0.71	0.66	0.62	0.51	0.56
O.....	20.42	20.36	20.37	20.46	20.32	20.85	20.05
Preparation No.	8.	9.	10.	11.	12.	13.	Average
C.....	55.07	55.19	55.06	55.03	54.89	55.04	55.19
H.....	7.47	7.40	7.41	7.30	7.46	7.43	7.36
N.....	16.53	16.64	16.58	16.45	16.45	16.51	16.44
S.....	0.64	0.50	0.60	0.59	0.60	0.65	0.60
O.....	20.29	20.27	20.35	20.63	20.60	20.37	20.41

*Distribution of Nitrogen in Kafirin.*—The distribution of nitrogen was obtained from an analysis made by the Van Slyke method. A sample of kafirin containing 16.64 per cent of nitrogen gave the

following results, from which it appears that this protein differs from zein in containing distinctly more amide nitrogen as well as basic nitrogen.

N	Kafirin.	Zein.*
Humin.....	0.17	0.16
Amide.....	3.46	2.97
Basic.....	1.04	0.49
Non-basic.....	11.97	12.51
Total.....	16.64	16.13

\*Osborne and Harris, *J. Am. Chem. Soc.*, 1903, xxv, 323.

The percentage of diamino-acids in kafirin was also determined by the Van Slyke method. The results given below have been corrected for 0.78 per cent of cystine which was precipitated with the phosphotungstates of the other bases.

	Kafirin.	Zein.*
Arginine.....	1.58	1.55
Lysine.....	0.90	0.00
Histidine.....	1.00	0.82
Tryptophane.....	Present.	0.00

\*See reference 2.

These results indicate that kafirin contains the lysine and tryptophane complexes, both of which are wholly lacking from zein.

#### SUMMARY.

A new alcohol-soluble protein has been isolated from kafir seeds, which constitutes more than one-half the protein in the seed. This protein has been named *kafirin*. Kafirin resembles zein in its ultimate composition, but is different in physical properties. It contains tryptophane and apparently lysine, both of which are lacking in zein. An analysis of kafirin has been made by the Van Slyke method. This shows quantitative differences between kafirin and zein.



# SOME PROTEINS FROM THE JACK BEAN, *CANAVALIA* *ENSIFORMIS*.\*

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Washington.)

(Received for publication, October 5, 1916.)

As far as we have been able to learn no attempts have been made to isolate any of the proteins from the jack bean. Barnstein<sup>1</sup> states that the ripe seed contains about 25 per cent of crude protein. We found that the seed with which we experimented contained 23 per cent of protein. When the jack beans were ground to a meal, distilled water extracted 15 per cent of protein, while a 2 per cent sodium chloride solution extracted 18.5 per cent, and a 10 per cent solution extracted 18 per cent. A 0.2 per cent solution of potassium hydroxide extracted nearly all of the protein or 22.3 per cent. When the meal was mixed with three times its weight of 10 per cent sodium chloride solution and this mixture ground in a mill to break up the cells, 20.5 per cent of protein were extracted ( $N \times 6.25$ ).

When sodium chloride extracts of the jack bean were dialyzed against distilled water, about 10 per cent of pure dry globulin, based on the weight of the meal used, was obtained. The globulin was so soluble in salt solutions that it could not be precipitated by diluting these solutions with water, although small quantities were obtained by saturating aqueous extracts of the jack bean with carbon dioxide. After the globulin had been removed the dialysate yielded about 1.5 per cent of an albumin. This was obtained by heating the solution to 80° C.

It is evident that the globulin of the jack bean is not identical with phaseolin from the kidney bean, *Phaseolus vulgaris*. The dif-

\* A preliminary report of this work was made at the Urbana meeting of the American Chemical Society (*Science*, 1916, xliv, 217).

<sup>1</sup> Barnstein, F., *Landw. Vers.-Stat.*, 1914, lxxxv, 113.



ference in composition of the globulins of the jack bean and phaseolin from the kidney bean is shown by the figures given below.

	Globulin from the jack bean.	Phaseolin from the kidney bean.*
C.....	52.94	52.66
H.....	6.96	6.93
N.....	16.49	15.83
S.....	0.45	0.36
O.....	23.16	24.22

\* Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 115.

Fractional precipitation of the globulin obtained from the jack bean by means of ammonium sulfate enabled us to isolate two globulins which differed chiefly in their sulfur content and their solubilities in ammonium sulfate solutions. The globulin obtained in the greatest amount and which was the most soluble and contained the least sulfur we have named *canavalin*. Associated with canavalin was a small quantity of a second globulin which was less soluble and had a much higher sulfur content than canavalin. To this globulin we have given the name *concanavalin*. These have the following composition.

	Canavalin.	Concanavalin.
C.....	53.26	53.28
H.....	7.03	7.02
N.....	16.72	16.45
S.....	0.48	1.10
O.....	22.51	22.15

Osborne and Campbell<sup>2</sup> found that the globulin precipitated by dialysis from extracts of the yellow lupine was a mixture of two globulins, conglutin A and conglutin B. These differed chiefly in their sulfur content, which was 0.52 and 1.65 per cent, respectively.

It is evident from the analysis of canavalin and concanavalin that only a small quantity of concanavalin can be present in the mixture of globulins obtained by dialysis, since the sulfur content

<sup>2</sup> Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1897, xix, 454. Osborne, T. B., and Harris, I. F., *Am. J. Physiol.*, 1905, xiii, 436.

of canavalin and the mixture of globulins is practically the same. The distribution of nitrogen in canavalin and phaseolin from the kidney bean is given below for comparison.

N	Canavalin.	Phaseolin.*
Humin.....	0.28	0.33
Amide.....	1.41	1.69
Basic.....	3.17	3.62
Non-basic.....	11.55	10.56
Total.....	16.41	16.20

\* Osborne, T. B., *The Vegetable Proteins*, London, 1909, 57.

The albumin present in extracts of the jack bean was easily obtained by coagulation after the globulins had been removed by dialysis. This protein resembled the legumelins which have been described by Osborne<sup>3</sup> and his coworkers. Its ultimate composition is given below.

C.....	53.24
H.....	7.00
N.....	16.38
S.....	0.88
O.....	22.50

The nitrogen in the albumin was distributed as follows:

Humin.....	0.23
Amide.....	1.16
Basic.....	3.73
Non-basic.....	11.18
Total.....	16.30

#### EXPERIMENTAL.

*Preparation of Meal from the Jack Bean.*—The air-dried beans were very hard and difficult to grind. They were therefore crushed by passing them through a power-driven meat chopper and then ground to a meal in an electrically driven coffee mill. This meal was placed in stoppered bottles and used, without further drying, for the experiments described below.

<sup>3</sup> Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 119.

*Globulins Extracted by Water and Precipitated by Carbon Dioxide.*—Aqueous extracts of jack bean meal were made by using 500 cc. of distilled water to each 100 gm. of meal. After stirring thoroughly to break up all lumps, the mixture was placed in an ice chest over night. It was then run through a peanut grinder to crush the swollen particles of meal, thereby obtaining a more complete extraction. The liquid so obtained was mixed with enough filter paper scrap to make a thick pulp. This was placed in cloths and pressed out with a powerful press. The turbid extract was very difficult to filter but was finally obtained in a clear form by filtering through paper pulp on a Buchner funnel. This filtrate was saturated with carbon dioxide. At first the solution became turbid and on standing over night in a cool place a part of the globulins subsided. Since the subsidence was not sufficient to permit separation by decantation, the whole mixture was filtered through a folded filter. The residue was washed with water and alcohol. It was then suspended in absolute alcohol for 24 hours, and after filtering off the alcohol it was suspended in absolute ether for the same length of time. After filtering off the ether the globulin was placed over sulfuric acid in a vacuum desiccator. It was finally dried in a vacuum oven at 110°C., the temperature being raised gradually. Two preparations made in this manner gave the following results on analysis.

	Preparation 1.				Preparation 2.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	52.14	52.15	52.15	52.51	52.71	52.64	52.67	53.02
H.....	6.78	6.75	6.76	6.82	6.78	6.92	6.85	6.94
N.....	16.42	16.46	16.44	16.55	16.89	16.98	16.93	17.04
S.....	0.47		0.47	0.47	0.44		0.44	0.44
O.....				23.65				22.56
Ash.....	0.66		0.66		0.57		0.57	

*Globulins Extracted by Sodium Chloride Solutions and Precipitated by Dialysis.*—An extract was made with a 2 per cent sodium chloride solution, using 300 gm. of the meal and 600 cc. of the salt solution. The extract was pressed out and filtered as described

above. The clear filtrate was then dialyzed in a parchment bag suspended in running distilled water until the chlorides had been removed. The globulin separated in the form of minute spheroids. These were dehydrated in absolute alcohol and ether, and finally dried to 110°C. in a vacuum oven. An analysis gave the following results.

	Preparation 3.	
		Ash-free.
C.....	52.03	53.03
H.....	6.92	7.04
N.....	16.23	16.53
S.....	0.39	0.40
O.....		23.00
Ash.....	1.87	

*Globulins Precipitated by Ammonium Sulfate.*—Preparations 4 to 6 were made by extracting the meal with 2 per cent sodium chloride solutions and saturating the clear filtrate with ammonium sulfate. The precipitate so produced was filtered off and redissolved in a small quantity of distilled water, since enough ammonium sulfate adhered to the protein to effect solution. A slight turbidity was removed by filtering through paper pulp and the filtrate was dialyzed until free from sulfates. Preparation 7 was obtained in the same manner except that the extract of the meal was made with a 10 per cent sodium chloride solution. These preparations gave the following results.

	Preparation 4.				Preparation 5.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	52.34	52.39	52.36	52.83	52.51	52.47	52.49	52.78
H.....	6.76	6.94	6.85	6.91	6.91	6.83	6.87	6.90
N.....	16.06	15.91	15.98	16.13	16.31	16.32	16.31	16.41
S.....	0.36		0.36	0.36	0.46		0.46	0.46
O.....				23.77				23.45
Ash.....	0.89		0.89		0.54		0.54	

	Preparation 6.		Preparation 7.	
		Ash-free.		Ash-free.
C.....	53.08	53.42	52.67	53.02
H.....	7.10	7.14	6.93	6.98
N.....	16.27	16.38	16.30	16.41
S.....	0.57	0.57	0.46	0.46
O.....		22.49		23.13
Ash.....	0.61		0.65	

*Summary of Preparations 1 to 7.*

	Preparation No.							
	1.	2.	3.	4.	5.	6.	7.	Average.
C.....	52.51	53.02	53.03	52.83	52.78	53.42	53.02	52.94
H.....	6.82	6.94	7.04	6.91	6.90	7.14	6.98	6.96
N.....	16.55	17.04	16.53	16.13	16.41	16.38	16.41	16.49
S.....	0.47	0.44	0.40	0.36	0.46	0.57	0.46	0.45
O.....	23.65	22.56	23.00	23.77	23.45	22.49	23.13	23.16

*Fractional Precipitation of the Globulins with Ammonium Sulfate.*

*Concanavalin.*—An extract was made from 1 kilo of jack bean meal, using 3 liters of a 1 per cent sodium chloride solution. The clear filtrate was made 0.6 saturated with ammonium sulfate. When this mixture was allowed to stand over night a flocculent precipitate settled. This was filtered off and redissolved by adding water, since enough ammonium sulfate was held by the residue to effect solution. A trace of insoluble substance was removed by filtering. The filtrate was then dialyzed until sulfates were removed. The small quantity of globulin which separated was prepared for analysis as previously described and gave Preparation 8. Another preparation (9) was obtained by extracting 1 kilo of the meal with a 2 per cent sodium chloride solution. The precipitate was filtered off and redissolved by adding water. This solution was then dialyzed until free from sulfates. Analyses of these preparations gave the following results.

	Preparation 8		Preparation 9	
		Ash-free.		Ash-free
C.....	53 34	53 58	52.73	52.98
H.....	6 93	6.96	7.04	7.08
N.....	16 58	16 65	16 17	16.25
S.....	1 13	1.13	1.06	1.07
O.....		21 68		22.62
Ash.....	0 45		0.43	

*Preparation of Canavalin.*—One preparation (10) of this protein was obtained from the filtrate from Preparation 8. After the concanavalin had been removed as previously described, the filtrate which was now 0.6 saturated with ammonium sulfate was made completely saturated by adding a slight excess of the same salt. The large quantity of precipitate which separated was filtered off and redissolved by the addition of water. After filtering clear, this solution was dialyzed to remove sulfates. Two other preparations (11 and 12) of canavalin were prepared in practically the same manner. These preparations were analyzed with the following results.

	Preparation 10		Preparation 11.		Preparation 12.	
		Ash-free		Ash-free		Ash-free
C	52 38	53 38	52 67	53 23	52 85	53 18
H	6 92	7 05	6 98	7 06	6 89	6 94
N.	16 52	16 83	16 47	16 64	16 63	16 74
S.	0 47	0 48	0 48	0 48	0 47	0 47
O		22 26		22 59		22 67
Ash	1 76		1 00		0 62	

*Distribution of the Nitrogen in Canavalin.*—Determinations to ascertain the distribution of the nitrogen in canavalin were made on two samples containing 16.40 and 16.42 per cent of nitrogen respectively. These samples probably contained a very small quantity of concanavalin, but not enough to affect the results of the analyses appreciably. The values obtained were as follows.

N	I.	II.	III.	Average.
Humin.....	0.27	0.28	0.30	0.28
Amide.....	1.30	1.43	1.50	1.41
Basic.....	3.08	3.15	3.28	3.17
Non-basic.....	11.75	11.56	11.34	11.55
Total.....	16.40	16.42	16.42	16.41

*Albumin from the Jack Bean.*—After the globulin had been removed from the sodium chloride extracts of the jack bean as completely as possible by dialysis the clear filtrate was heated to obtain any albumin that might be present. A turbidity appeared at 50°C. and a coagulum at 62°C. The temperature was raised to 65°C. and the heating continued until no more coagulum formed. The filtrate from this coagulum showed a slight turbidity at 75–80°C. and gave but a trace of coagulum between 85 and 95°C. Preparations of the albumin were made by heating solutions, from which the globulin had been removed, at 75–80°C. In this way a white coagulum was obtained. This was filtered off, washed with water, and suspended in alcohol and finally in ether. The albumin thus obtained was a white powder, and analyses of four different preparations gave results which agreed almost within the limits of error of the analytical methods. The results of these analyses follow.

	Preparation 13.		Preparation 14.		Preparation 15.		Preparation 16.	
		Ash-free.		Ash-free.		Ash-free.		Ash-free.
C.....	52.69	53.28	53.08	53.28	52.88	53.26	52.76	53.13
H.....	7.03	7.10	6.96	6.99	6.87	6.96	6.93	6.98
N.....	16.13	16.31	16.33	16.42	16.24	16.35	16.34	16.45
S.....	0.81	0.81	0.97	0.97	0.86	0.86	0.86	0.86
O.....		22.50		22.34		22.57		22.58
Ash.....	1.00		0.36		0.64		0.64	

The nitrogen in the albumin from the jack bean was distributed as follows:

Humin .....	0.23
Amide.....	1.16
Basic.....	3.73
Non-basic .....	<u>11.18</u>
Total.....	16.30

## SUMMARY.

Two globulins, canavalin and concanavalin, and an albumin of the legumelin type have been isolated from the jack bean. The distribution of nitrogen in canavalin and in the albumin has been determined.





# THE PROTEINS OF THE PEANUT, *ARACHIS HYPOGÆA*.

## I. THE GLOBULINS ARACHIN AND CONARACHIN.

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(Received for publication, October 13, 1916.)

The ravages of the boll weevil have made it unprofitable to grow cotton in various sections throughout the South. In many regions where the growing of cotton has been decreased, peanuts are now being grown as a supplementary crop. This has caused a large increase in the production of peanuts in the United States. A number of mills which in the past produced cottonseed oil are now using their presses for the production of peanut oil. The press-cake thus obtained as a by-product is used for cattle food and sells at about thirty-five dollars a ton. This cake, therefore, furnishes a relatively cheap source of food with a very high protein content.

The most striking result brought out during this investigation is the fact that the globulins of the peanut contain a high percentage of basic nitrogen when compared with the proteins of other seeds commonly used for food. Work on the hydrolysis and separation of the amino-acids of the peanut globulins is in progress and will be reported in a later publication.

The proteins of the peanut have received but little attention. The only published experiments seem to be those described by Ritthausen<sup>1</sup> in a paper which appeared in 1880. This author extracted oil-free peanut meal with a solution of sodium chloride and with solutions of potassium, calcium, and barium hydroxides. From the sodium chloride extract he obtained the globulin by saturating the diluted extract with carbon dioxide, or simply by diluting the extract with a large volume of water. Apparently the same globulin was obtained by acidifying the alkaline ex-

<sup>1</sup> Ritthausen, H., *Arch. ges. Physiol.*, 1880, xxi, 81.

tracts with acetic or sulfuric acids. Ritthausen found no evidence in his analytical data that indicated the presence of more than one globulin in the peanut.

The peanut meal with which Ritthausen experimented had been dried over sulfuric acid in a desiccator and contained 10.18 per cent of nitrogen or 56 per cent of protein. The latter value was obtained by multiplying the percentage of nitrogen by 5.5, thus assuming that the protein contained 18 per cent of nitrogen. When this meal was extracted with 10 per cent sodium chloride solution he isolated 27 per cent of globulin by diluting the extract with water and then saturating with carbon dioxide.

We have extracted air-dried oil-free peanut meal containing 42 per cent of protein ( $N \times 5.5$ ) with a 10 per cent sodium chloride solution and have found that about 32 per cent of protein, based on the weight of meal used, is dissolved at room temperature. When the temperature was raised to 40–50°C. the yield was not increased. From a sodium chloride extract of the meal, calculated to contain 32 gm. of globulin, we were able to isolate about 25 gm. by diluting the sodium chloride extract with water or by saturating with carbon dioxide. This yield is based on the actual weight of pure protein after drying at 100°C. and does not take into consideration the losses incurred during its isolation.

The analyses of ten preparations of peanut globulin precipitated by dilution or by dialysis of sodium chloride extracts gave results which agree well with those published by Ritthausen, except the value for carbon, which we found to be about 0.7 per cent higher than that reported by him.

We have made fractional precipitations of the protein extracted by salt solutions from peanut meal and in this manner have isolated two globulins, one of which is present only to a small extent. The globulin composing the greater part of the precipitate obtained by diluting sodium chloride extracts of peanut meal with water is the less soluble of the two and is precipitated, when in 10 per cent salt solution, by adding ammonium sulfate to 0.2 of saturation. To this globulin we have given the name *arachin*. After filtering off the *arachin* the second globulin may be obtained by dialysis or by saturating the filtrate with ammonium sulfate. This second globulin we propose to call *conarachin*. The differ-

ence in composition of these two globulins is shown by the following analyses.

	Arachin.	Conarachin.
C.....	52.15	51.17
H.....	6.93	6.87
N.....	18.29	18.29
S.....	0.40	1.09
O.....	22.23	22.58

It will be seen that the greatest difference between these two globulins is in the percentage of sulfur, which is nearly three times as great in conarachin as in arachin. Another striking difference between these two proteins is shown by the figures representing the distribution of the nitrogen. The figures are as follows:

N	Arachin.	Conarachin.
Amide.....	2.03	2.07
Humin.....	0.22	0.22
Basic.....	4.96	6.55
Non-basic.....	11.07	9.40
Total.....	18.28	18.24

The difference in the basic nitrogen as shown by these results is most striking for, as far as we know, conarachin contains more basic nitrogen than any other seed globulin yet examined. The percentage of basic nitrogen in the mixture of these globulins is also high, being 5.23 per cent. For the sake of comparison the percentages of basic nitrogen in some proteins from seeds commonly used for foods are given below.<sup>2</sup>

<sup>2</sup> Osborne, T. B., *The Vegetable Proteins*, London, 1909, 57.

Protein.	Source.	Basic nitrogen.
		<i>per cent</i>
Zein.....	Maize.	0.49
Gliadin.....	Rye.	0.91
".....	Wheat.	1.09
Phaseolin.....	Kidney bean.	3.62
Vicilin.....	Pea.	4.92
Arachin.....	Peanut.	4.96
Legumin.....	Pea.	5.11
Excelsin.....	Brazil nut.	5.76
Edestin.....	Hempseed.	5.97
Globulin.....	Cocoanut.	6.06
Conarachin.....	Peanut.	6.55

Besides arachin and conarachin, sodium chloride solutions extract from peanut meal a trace of albumin which coagulates at 65–70°C. The quantity of albumin is so small that we have not yet been able to obtain a pure product in sufficient amounts for a complete analysis. Carbon, hydrogen, and nitrogen determinations made on a small quantity of this albumin gave results which agree closely with the values obtained by Osborne and his coworkers<sup>3</sup> for the legumelins frequently found in the seeds of leguminous plants.

If the diamino-acids represented by the basic nitrogen of the peanut proteins are present in the proper proportions, peanut press-cake may be used to supplement the deficiency of basic nitrogen in such foods as wheat, oats, and corn. Mixtures of these cereals with peanut press-cake or peanut flour may prove to be a highly nutritious diet and may be used as food for human beings as well as animals at less cost than when the cereals are used alone.

Investigations on the proteins of the peanut will be continued.

#### EXPERIMENTAL.

*Preparation of Meal from the Peanut.*—Shelled Virginia peanuts of the best quality were used in this investigation. The

<sup>3</sup> Osborne, *Ergebn. Physiol.*, 1910, x, 126.

peanuts were pressed cold in an Anderson expeller to remove most of the oil. The resulting press-cake was then ground to a meal, the residual oil was removed by means of cold petroleum ether, and after filtering by suction the meal was placed in a vacuum desiccator until free from petroleum ether.

*Globulins Precipitated by Diluting Sodium Chloride Extracts of Peanut Meal with Water.*—In extracting the globulins from peanut meal 500 cc. of a 10 per cent sodium chloride solution were used for each 100 gm. of meal. The meal was stirred into the salt solution, giving a thick pasty mass. This paste was run through a grinding mill three times and a thin mixture was obtained as the distended cell walls were broken up. Dry filter paper scrap was then added and the mixture was worked up with the hands until a rather stiff pulp was obtained. This was placed in cloths and the liquid forced out by means of a powerful press. In this way we pressed out a volume of extract equal to about 80 per cent of that of the sodium chloride solution used. The press juice was then filtered clear by suction through a thick mat of paper pulp and a clear fluorescent liquid was obtained. This was diluted by adding five or six volumes of distilled water. The globulins precipitated immediately in the form of a viscous paste, which, when stirred with a rod, exhibited a silvery sheen. The supernatant liquid was poured off and a little distilled water added. On stirring with a glass rod the character of the precipitate was altered so that it was converted into a finely divided granular substance and an opaque milk-like fluid. The latter could neither be filtered nor caused to separate by centrifugating. The addition of alcohol at this stage usually caused the milky liquid to separate so that it could be filtered off by suction or thrown down by centrifugating. The precipitate thus produced was washed with 50 per cent alcohol and then dried by suspending it in absolute alcohol for 24 hours. The protein was then filtered off and suspended in anhydrous ether for another 24 hours. After filtering off the ether the protein was placed in a vacuum desiccator and was finally dried in a vacuum oven by raising the temperature gradually to 110°C. The globulin was thus obtained as a white powder. Analyses of five different preparations are given below.

	Preparation 1.		Preparation 2.			
		Ash-free.	I.	II.	Average.	Ash-free.
C.....	51.48	52.11	51.48	51.35	51.41	52.06
H.....	6.61	6.69		6.63	6.63	6.71
N.....	18.01	18.22	18.05		18.05	18.27
S.....	0.46	0.46	0.42		0.42	0.43
O.....		22.52				22.53
Ash.....	1.18		1.22		1.22	

	Preparation 3.				Preparation 4.	
	I.	II.	Average.	Ash-free.		Ash-free.
C.....	51.42	51.52	51.47	51.87	52.10	52.53
H.....	6.76	6.70	6.73	6.78	6.74	6.79
N.....	18.19	18.23	18.21	18.34	18.17	18.32
S.....	0.48		0.48	0.48	0.61	0.61
O.....				22.53		21.75
Ash.....	0.78		0.78		0.80	

	Preparation 5.			
	I.	II.	Average.	Ash-free.
C.....	51.54	51.78	51.66	52.11
H.....	6.65	6.74	6.69	6.72
N.....	17.87		17.87	18.03
S.....	0.41		0.41	0.41
O.....				22.73
Ash.....	0.87		0.87	

*Globulins Obtained by Dialysis of Sodium Chloride Extracts of Peanut Meal.*—Sodium chloride extracts of peanut meal were prepared as described above. These extracts were dialyzed in parchment bags, suspended in running water, until the solution ceased to give a test for chlorine. Toluene was used to keep the solution sterile. The analyses of three preparations thus obtained are given below. It will be seen that these preparations differ but slightly from those obtained by precipitating the globulins by adding water to the sodium chloride extract.

	Preparation 6.				Preparation 7.				Preparation 8.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	51.91	52.11	52.01	52.39	52.04	52.12	52.08	52.40	51.37	51.32	51.34	51.60
H.....	6.57	6.76	6.66	6.71	6.67	6.75	6.71	6.75	6.68	6.64	6.66	6.69
N.....	18.01	18.10	18.05	18.24	17.77	17.87	17.82	17.92	17.95		17.95	18.05
S.....	0.50		0.50	0.50	0.48		0.48	0.48	0.67	0.62	0.64	0.64
O.....				22.16				22.45				23.02
Ash...	0.76		0.76		0.59		0.59		0.55		0.55	

*Globulins Obtained by Saturating Sodium Chloride Extracts of Peanut Meal with Carbon Dioxide.*—Sodium chloride extracts of peanut meal were diluted with water until a faint turbidity was produced and were then saturated by passing a stream of carbon dioxide through the liquid. A compact white precipitate was obtained on allowing the mixture to settle over night. Two preparations made in this way gave the results stated below.

	Preparation 9.				Preparation 10.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	52.17	52.85	52.01	52.17	52.21	52.00	52.10	52.29
H.....	6.81	6.75	6.78	6.79	6.51	6.71	6.61	6.63
N.....	18.17	18.21	18.19	18.24	18.08		18.08	18.15
S.....	0.49		0.49	0.49	0.42		0.42	0.42
O.....				22.31				22.51
Ash.....	0.29		0.29		0.34		0.34	

*Summary of Analyses of the Total Globulin Obtained from the Peanut.*

Preparation No.....	1.	2.	3.	4.	5.	6.
C.....	52.11	52.06	51.87	52.53	52.11	52.39
H.....	6.69	6.71	6.78	6.79	6.72	6.71
N.....	18.22	18.27	18.34	18.32	18.03	18.24
S.....	0.46	0.43	0.48	0.61	0.41	0.50
O.....	22.52	22.53	22.53	21.75	22.73	22.16
Preparation No.....	7.	8.	9.	10.	Average.	Ritt-hausen.
C.....	52.40	51.60	52.17	52.29	52.15	51.41
H.....	6.75	6.69	6.79	6.63	6.72	6.74
N.....	17.92	18.05	18.24	18.15	18.18	18.25
S.....	0.48	0.64	0.49	0.42	0.50	0.56
O.....	22.45	23.02	22.31	22.51	22.45	23.04



*Nitrogen Distribution in the Total Globulin in the Peanut.*—The distribution of the nitrogen in the total globulin obtained from the peanut was found by Hausmann's method as modified by Osborne and Harris. Results obtained on two different preparations (a and b) are given below. The calculations were made on an ash- and moisture-free basis.

N	Preparation a.			Preparation b.		
	I.	II.	Average.	I.	II.	Average.
Amide.....	2.05	2.10	2.08	2.07	2.09	2.08
Humic.....	0.18	0.24	0.21	0.21	0.19	0.20
Basic.....	5.29	5.22	5.25	5.18	5.25	5.21
Non-basic.....	10.63	10.59	10.61	10.78	10.71	10.75
Total.....	18.15	18.15	18.15	18.24	18.24	18.24

*Fractional Precipitation of the Globulins of the Peanut.*

*Isolation of Arachin.*—An extract was made as previously described, using 500 gm. of peanut meal and 2.5 liters of a 10 per cent sodium chloride solution. To the clear filtrate solid ammonium sulfate was added gradually until the solution became 0.2 saturated with this salt. A precipitate began to appear at 0.15 of saturation and increased rapidly until 0.2 of saturation was reached, when precipitation practically ceased and did not occur again until much more ammonium sulfate was added. On allowing the 0.2 saturated solution to stand over night, the precipitate settled out in a very compact form so that the supernatant liquid could be easily decanted. The precipitate was washed with a 10 per cent sodium chloride solution containing ammonium sulfate to 0.2 of saturation. The residue was then redissolved in a small volume of 10 per cent sodium chloride, and the resulting solution was filtered and dialyzed until chlorides were removed. In this manner the greater part of the globulin present in the meal was obtained as a white powder. This was dried for analysis in the manner previously described. Three different preparations gave the following results.

	Preparation 11.				Preparation 12.				Preparation 13.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	52.08	52.25	52.16	52.51	51.55	51.53	51.54	51.86	51.90	51.84	51.87	52.16
H.....	6.96	6.98	6.97	7.02	6.96	6.81	6.88	6.93	6.95	6.80	6.87	6.91
N.....	18.16	18.12	18.14	18.26	18.22	18.27	18.24	18.37	18.17		18.17	18.28
S.....	0.37		0.37	0.37	0.41		0.41	0.41	0.44		0.44	0.44
O.....				21.84				22.43				22.21
Ash....	0.69		0.69		0.62		0.62		0.57		0.57	

*Distribution of the Nitrogen in Arachin.*—The sample used for this experiment contained 18.28 per cent of nitrogen, calculated on an ash- and moisture-free basis. The results are given below.

N	I.	II.	Average.
Amide.....	2.06	2.00	2.03
Humins.....	0.23	0.20	0.22
Basic.....		4.96	4.96
Non-basic.....		11.12	11.07
Total.....		18.28	18.28

*Isolation of Conarachin.*—The filtrate from Preparation 12, from which arachin had been removed, as described above, and which was now 0.2 saturated with ammonium sulfate, was completely saturated by adding more ammonium sulfate. This treatment produced a small quantity of precipitate. This was redissolved in 10 per cent sodium chloride and the filtered solution dialyzed until free from chlorides. A small quantity of conarachin, Preparation 14, was obtained. Another preparation of conarachin was made by dialyzing the filtrate from No. 13. This was designated No. 15. These preparations gave the following results on analysis.

	Preparation 14.				Preparation 15.			
	I.	II.	Average.	Ash-free.	I.	II.	Average.	Ash-free.
C.....	50.89	51.12	51.00	51.16	50.95	51.03	50.99	51.19
H.....	6.86	6.82	6.84	6.87	6.77	6.89	6.83	6.86
N.....	18.34	18.24	18.29	18.34	18.13	18.22	18.17	18.24
S.....	1.07	1.05	1.06	1.06	1.12		1.12	1.12
O.....				22.57				22.59
Ash....	0.32		0.32		0.37		0.37	



# A MODIFIED BENEDICT AND HITCHCOCK URIC ACID STANDARD SOLUTION.

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It was repeatedly observed during the winter months in this laboratory that from the standard solution of uric acid prepared according to the directions of Benedict and Hitchcock,<sup>1</sup> crystals of uric acid would separate out within a week or two after its preparation, thus rendering the standard useless. It was suspected that the low temperature of our laboratory was responsible for the crystallization, particularly as a deposit of uric acid was noticed in the morning following an extremely cold night or after a period of moderately cold weather when the laboratory temperature during the night was considerably reduced. Even when a standard solution was kept in the hospital laboratory where the temperature during the greater part of the day was normal, it was found one morning following a cold night, that crystallization had set in. It is probable that many others have had a similar experience. It appears, therefore, that the uric acid standard proposed by Benedict and Hitchcock, however reliable in mild or warm weather, does not meet the requirements for winter work.

After a great many experiments, we have found that by the substitution of boric for acetic acid, a reliable standard is obtained which does not crystallize in cold weather.

## EXPERIMENTAL.

As the quantity of acetic acid is probably the controlling factor in causing the crystallization of uric acid, it was thought

<sup>1</sup> Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 623.

that by diminishing the amount of acid, a stable standard would be obtained. To this end three standard solutions of uric acid were prepared following the directions of Benedict and Hitchcock, containing respectively 1.4, 1.3, and 1.2 cc. of glacial acetic acid. On the same day, to determine the most favorable amount of boric acid which could be substituted for the acetic acid, three other standard solutions of uric acid were prepared containing 25, 37, and 50 cc., respectively, of 4 per cent boric acid in place of the acetic acid. The six standard solutions were kept in the laboratory and tested from time to time against a freshly prepared pyridine-uric acid solution made up in accordance with the directions supplied by Benedict and Hitchcock.<sup>2</sup> According to these authors 1 cc. of the pyridine-uric acid solution, containing 1 mg. of uric acid, is taken as a standard for comparison; while with their phosphate-uric acid solution, 5 cc., containing 1 mg. of uric acid, are employed. Following the directions of Benedict and Hitchcock, we were unable to obtain uniformly concordant results when freshly prepared pyridine and phosphate solutions were compared. This we believe is due to the large error entailed in the use of a 1 cc. pipette. The use of a graduated Mohr pipette gave no better results than those given by the usual volumetric type.

To minimize the error occasioned in measuring 1 cc. of a relatively strong solution, the following procedure was tried: By means of an accurate 10 cc. pipette, 10 cc. of the pyridine-uric acid solution were introduced into a 50 cc. volumetric flask and diluted to the mark. After thorough mixing, 5 cc. of the resulting diluted solution, containing 1 mg. of uric acid, were compared with an equal volume of the phosphate-uric acid solution. The results compared favorably. We therefore adopted this method of dilution in all the experiments reported in this paper. The uric acid in the six solutions was determined colorimetrically in accordance with the directions given by Benedict and Hitchcock. Extreme care was taken to keep the conditions as uniform as possible throughout the determinations. The following results were obtained.

<sup>2</sup> Benedict and Hitchcock, *J. Biol. Chem.*, 1915, xx, 622.

TABLE I.  
Solutions Prepared February 23, 1916.

Time of testing.	Standard uric acid solutions.					
	Containing acetic acid (B. and H.).			Containing boric acid (C. and F.).		
	Acetic acid.			4 per cent boric acid.		
	1.2 cc.	1.3 cc.	1.4 cc.	25 cc.	37 cc.	50 cc.
	cc.	cc.	cc.	cc.	cc.	cc.
Feb. 24.....	19.8	19.7	19.8	19.9	19.9	19.7
" 28.....	19.7	20.5*	20.2*	19.9	19.9	19.5
Mar. 2.....	21.2†	22.5†	27.8†	19.9	19.9	19.9
" 18.....				19.9	20.0	19.9
" 27.....				19.9	20.0	20.0
Apr. 27.....				19.9	20.0	20.2
May 22.....	23.0	32.3	52.8‡	21.5	21.3	21.5

\* Slight deposit of uric acid was observed in this solution, Feb. 29.

† The standard solution contained a decided deposit of uric acid.

‡ The solution was so weak that the standard had to be set at 10.

The above figures are the average of at least five well agreeing readings and conclusively show that the phosphate-uric acid standard containing boric acid is superior in cold weather to that containing acetic acid. Analyses of the acetic acid standards after March 2 were not regularly made because of the copious crystallization which had taken place in all of them. It was thought desirable, however, when the last run was made on the 22nd, to determine the strength of the acetic acid standards for the purpose of ascertaining to what degree they had deteriorated. The determinations were made on 5 cc. portions of the clear supernatant solutions. The order of decomposition of the acetic acid standards as shown in the last figures is the same as that which is at once apparent in comparing the size of the uric acid precipitates on the bottom of the bottles. The colorimetric readings for the boric acid solutions show that they remained unchanged for 2 months and that at the end of 3 months a deterioration of 5 per cent had taken place. We feel on the basis of the above results that we can recommend the phosphate standard containing boric acid as a reliable standard for the winter months.

To determine whether or not the boric acid standard would retain its strength in warm weather, freshly prepared uric acid solutions containing boric and acetic acid respectively were kept in the laboratory during the summer months and analyzed at the time of preparation and again at the expiration of  $2\frac{1}{2}$  months. The results showed that for warm weather the boric acid standard is decidedly inferior to the acetic acid standard. None of the uric acid solutions containing acetic acid showed any signs of crystallization.

#### SUMMARY.

1. It has been shown that the Benedict and Hitchcock phosphate-uric acid solution is unreliable as a standard for the determination of uric acid in cold weather, owing to its tendency to crystallize.

2. By substituting boric for acetic acid, a standard was prepared which did not crystallize and showed no deterioration in 2 months of cold weather.

In conclusion, the authors wish to express their thanks to Dr. Isidor Greenwald of this laboratory for his interest and assistance.

# THE OCCURRENCE AND PHYSIOLOGICAL SIGNIFICANCE OF FLAVONE DERIVATIVES IN PLANTS.

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## PLATE 1.

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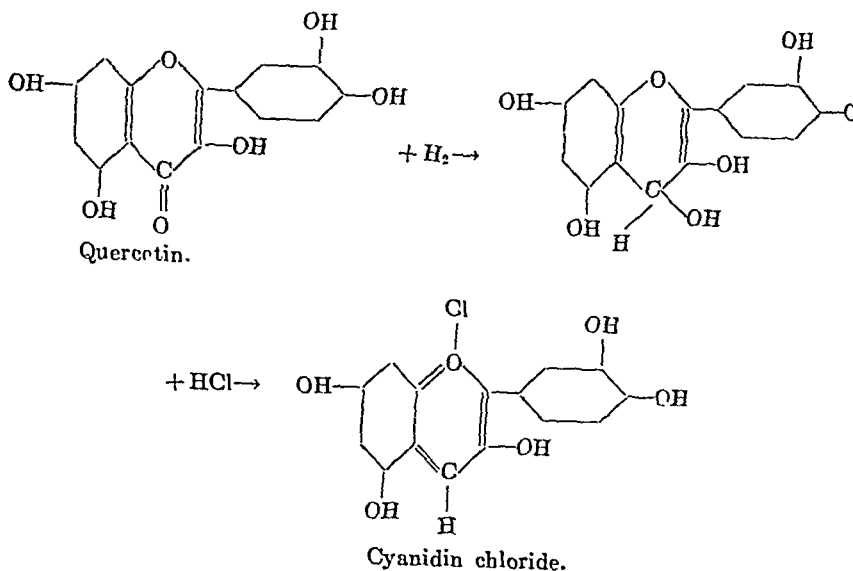
## INTRODUCTION.<sup>1</sup>

We owe to Willstätter and his collaborators, through their chemical investigations, definite evidence bearing upon the relationship between two classes of naturally occurring plant pigments, the anthocyanins and the yellow substances which are of the flavone or flavonol class. It has been established by them<sup>2</sup> that cyanidin from the corn flower is in reality a reduction product of quercetin, which is already known as one of the synthetic flavonols. The reaction is expressed in the following equation:

<sup>1</sup> See also the following papers: Shibata, K., *Bot. Magazine* (Tokyo), 1915, xxix, 118. Shibata, K., and Kishida, M., *ibid.*, 1915, xxix, 301. Shibata, K., and Nagai, I., *ibid.*, 1915, xxx, 149. For the detailed account and bibliography the reader should refer to the original papers.

<sup>2</sup> Willstätter, R., and Mallison, H., *Sitzungsber. Preuss. Akad. Wiss.*, 1914, xxix, 769.





Further investigations, extended to other anthocyanins isolated from different flowers and fruits, definitely established the molecular structures of these pigments. The anthocyanins are now understood to occur in plants as glucosides, of which the color-bearing components (anthocyanidins) are the reduction products of flavonols and the combined sugar is either glucose or galactose. Thus by hydrolysis, cyanin, the anthocyanin pigment from the corn flower, yields one molecule of cyanidin and two molecules of glucose; likewise idaein, the pigment of the cranberry, yields one molecule of cyanidin and one molecule of galactose.

Previously, Everest has shown that flavone and flavonol glucosides, which are contained in the extract from the yellow flower, produce the anthocyanin-like red substance by reduction without removal (hydrolysis) of the combined sugar.

This chemical evidence has led us to undertake a series of investigations on the physiological and biological significance of these substances in plants. The evidence brought forward establishes a somewhat unexpected fact; namely, the flavone derivatives are one of the cell contents of very common occurrence in the plant kingdom, in fact, they are quite as common

as chlorophyll, tannins, carotinoids, sugars, starches, and proteins. Flavone derivatives are found not only in the yellow coloring matter, as in the cases already reported, but also in the cell sap of the epidermis and the underlying tissue of plants in general.

*The Formation of Anthocyanin in the Flower of Diervilla and Others.*

Our attention was at first directed to the formation of the anthocyanin pigment in the flower of *Diervilla grandiflora* S et Z, a flowering shrub commonly found in fields and gardens in our vicinity. A characteristic feature of this flower is a rapid formation of red color. The corolla is snow-white at the time of blooming, but it is tinged gradually with pale rose color, and at the end of the day of blooming the corolla is completely changed to a beautiful rose color. Thus a cluster of flowers on a single branch shows a gradation of color from white to deep rose, according to the age of the flower. A microscopic examination of a colored corolla reveals the fact that the deeply colored pigment occurs as a number of round droplets of different sizes imbedded in the paler colored cell sap of the epidermal cells. In the white corolla, however, none of them are observed.

A rapid formation of anthocyanin in the white *Diervilla* flower naturally suggests the possibility that the mother substance of the red pigment (chromogen) may be present in the white flower. The above assumption is justified by the fact that the colorless alcoholic extract from the white *Diervilla* flower produces purple-red anthocyanin solution by reduction. The method of reduction used is as follows:

5 to 10 cc. of the alcoholic extract, prepared by heating the white flower with alcohol, are acidified by an addition of five to ten drops of concentrated hydrochloric acid. A few cc. of the mixture are received in a test-tube with a drop of mercury the size of a pea, and a small amount of magnesium powder. Reduction takes place with a vigorous generation of hydrogen gas whereby the mixture becomes beautifully colored.

The red pigment thus produced from the colorless extract of the white flower of *Diervilla* is stable in acid solution and soluble in water and in alcohol. The chemical behavior and the absorption spectra of the pigment produced are the same as

those of the anthocyanin pigment extracted from the naturally colored corolla. Further, a white corolla gives a deep yellow color on exposure to ammoniacal vapor.<sup>3</sup> It is established, therefore, that the chromogen substance occurs in the white flower of *Diervilla* as a flavonol glucoside and that the production of anthocyanin is performed by the reduction of the same.

It is possible to detect the presence of flavones from the various parts of the plant by means of the reduction test<sup>4</sup> due to the production of the red color of the extract by reduction, together with an additional ammoniacal test, due to the production of yellow color as mentioned above. The reduction test can be performed in such a manner as to make a rough quantitative determination of the flavone content by comparing the depth of reduction color of the extract of known amount, with the standard color solutions.

By these two methods we have found that almost all kinds of white flowers, including the lily, daisy, foxglove, dahlia, petunia, primrose, phlox, iris, opuntia, tobacco, and pansy, contain flavone or flavonol glucosides. Only in a small number of plants is a complete absence of chromogen proved; for example, in the white corn flower (*Centaurea cyanus*), oxalis (*Oxalis violacea*), pink (*Dianthus caryophyllus*), and pelargonium (*Pelargonium cucullatum*).<sup>5</sup>

<sup>3</sup> Wheldale, M., *J. Genetics*, 1914, iv, 113.

<sup>4</sup> No other organic compounds examined, such as carbohydrates, glucosides, phenols, tannins, amines, ureides, amino-acids, polypeptides, proteins, nucleins, etc., give similar reduction color.

<sup>5</sup> With reference to flavones, several types of white flowers can be considered. (1) Those which contain no flavones (absence of chromogen). (2) Those containing such a class of flavones as chrysin (dihydroxyflavone), which produces no red but only a yellow color by reduction. (3) Those containing a class of flavones which produce only an orange-red but not a true anthocyanin color by reduction. (4) Those which contain a class of flavonols which produce a red anthocyanin color by reduction. (5) Those which contain a mixture of different classes of flavones and flavonols. (Compare page 100.) This relationship seems to be of considerable importance in the genetic studies of flower color. Two types are known to occur among the white flowers which differ in their genetic behavior when they are crossed with colored types. They are classified by genetists as "dominant white" and "recessive white." It seems possible that these genetic relations can be rendered in chemical terms by the joint studies of genetists and chemists in the future.

The presence of flavone derivatives is proved in other parts of the floral organs; *i.e.*, calyx, stamens, style, ovule, bracts, etc., and also in the vegetative organs; *i.e.*, leaves, shoots, etc., as has already been mentioned. The occurrence of flavones in the green leaves can be proved in a simple manner. 1 to 2 gm. of fresh or dried material are cut up in small pieces and the extraction is made either by hot alcohol or boiling water. In the case of fresh material water is used, but in the case of dried material, hot alcohol is always preferable. In the latter case, the extracted chlorophyll does not generally interfere with the reaction, being itself readily reduced to an almost colorless product. If necessary, however, it can first be removed from the extract by shaking with petroleum ether. The reduction of the extract is carried out as described above.<sup>6</sup> In this way positive proof of the occurrence of flavone derivatives is obtained in the frond of marine algæ (*Dictyota dichotoma* and some other brown algæ), in the thallus of mosses, in the leaves and shoots of ferns, cycads, conifers, angiosperms, even in the scales of the winter buds, and in the resinous excretions on the surface of the bud scales of the poplar, horse-chestnut, alder, rhododendron, etc.

#### *The Physiological Significance of the Flavone Derivatives in Plants.*

The question now arises as to the physiological and biological significance attached to the flavone derivatives which are, by the present studies, proved to be substances of common occurrence in the plant tissue. In view of the fact that the presence of those substances is localized chiefly in the epidermis and in the peripheral layer of the tissue of the aerial part of the plant, and further, that plants grown in the shade or under a glass covering contain but small amounts, it seems that at least one

<sup>6</sup> The result we obtained does not agree with that of Everest (*Proc. Roy. Soc., Series B*, 1914, lxxxvii, 450). He states, "This method (*i.e.*, reduction by magnesium and hydrochloric acid) was of no value when working with crude plant extracts, as alcoholic extracts contained so much extraneous matter that the results were masked." His failure is due to the absence of mercury in the mixture, the use of this catalyzing agent being essential, as it was found, in carrying out the reduction test.

of the important functions of these substances might be related to the action of the sunlight.

It is shown that a solution of quercitrin (a flavonol glucoside) and apigenin (trioxyflavone) greatly diluted absorbs the ultra-violet region of the iron arc light almost completely, as shown in Figs. 1 and 2.

The above described absorption spectra of the dilute solutions of chemically pure flavone derivatives seem to show that the function of the flavone glucoside dissolved in the cell sap is to protect the living protoplasm and the important biochemical agents involved from the injurious action of the ultra-violet rays of sunlight by absorbing them at the peripheral layer of the plant organism.

Another possible significance of the flavone derivatives is the rôle they play in the respiration processes of plants. Remembering the peculiar chemical behavior of the substances resulting from the addition capacity of the pyrone oxygen, their function as hypothetical acceptor in Palladin's respiration scheme seems very suggestive.

The plausibility of the assumed function of flavone derivatives in plants as the chemical ray filter is amply proved. Plants in a sunny habitat are always richer in flavones than those growing in the shade. The aquatic plants, for example, *Nelumbo*, *Nuphar*, *Nymphaea*, *Trapa*, etc., which are exposed to direct and reflected sunlight over the surface of water, are especially rich in flavones. They occur abundantly in the aerial parts and in the case of the floating leaves only in the upper epidermis, while in the submerged and underground portions, only a trace is detected. Furthermore, an extensive observation of the flora of alpine and tropical regions has given us very positive evidence of this. In those regions, the insolation is much more intense than in the lower<sup>7</sup> or temperate regions<sup>8</sup> and the majority of the plants grown under such an environment are extraordinarily rich in flavones.

<sup>7</sup> According to Violle (cited in C. Schroeter's *Das Pflanzenleben der Alpen*, Zürich, 1904) the amount of insolation, calorimetrically measured, is 26 per cent stronger at the summit of Mont Blanc (4,810 meters) than in Paris. C. Dorno (*Studie über Licht u. Luft d. Hochgebirges*, 1911, reference in *Meteorol. Z.*, 1912, xx, 64) observed a seasonal change in the amount of

*The Flavone Content of Alpine Plants.*

Before entering into a discussion of this subject the method adopted for the comparative determination of the flavone content in the different plant material may be mentioned. Flavones are not lost appreciably if the drying is done quickly, so that the determination can be made with a small amount of any well preserved herbarium material, even that which has been kept for a number of years.

The extraction from the dried material is usually made by means of twenty parts of warm alcohol. To 4 cc. of the extract so prepared 1 cc. of 30 per cent hydrochloric acid is added, and the mixture is reduced by means of a drop of mercury and magnesium powder, which is added little by little to the mixture till no more color is developed. The intensity of color thus produced by reduction (anthocyanin) is compared with that of standard solutions, and the amount of flavone contained in the extract is thus determined. The standard solutions are provided by the alcoholic solutions of chemically pure flavones or flavonols of known concentration which are reduced in the same manner, as described elsewhere. The grades of color provided in the standard solutions are as follows:

Concentration of flavone.	Color scale.
1 : 1,000	I
1 : 2,000	II
1 : 3,000	III
1 : 5,000	IV
1 : 10,000	V
1 : 20,000	VI

---

ultra-violet rays of the sunlight in Davos (1,560 meters), Switzerland. In winter and spring, the amount is very low and suddenly rises in May. The maximum is reached in the months of July and August, and a sudden fall occurs in September. The growing season of the alpine plants, therefore, is in the months of maximum intensity of chemical sunlight in the year.

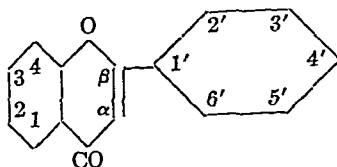
\* Data given by H. E. Roscoe (*Phil. Tr.*, 1867, clvii, 555) show that the chemical intensity of total daylight at Parà in 3 days in the month of April (1866) is about twenty times stronger than that at Kew. Even the intensity at Parà in April is still three and one-third times stronger than that at Kew in August.

The reduction color of different members in the flavone or flavonol series is by no means the same. In general the color produced by the flavone class is orange-red, whereas that produced by the flavonol class is red to violet-red. The reduction product of the various plant extracts also gives both types of color. Two sets of standard colors, therefore, are prepared from apigenin (A) and quercetin (Q) to make the comparison more exact.

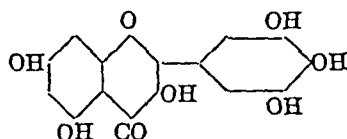
The reduction colors produced by the alcoholic solution of different flavones and flavonols are as follows:

Substance.*	Reduction colors.
<b>Flavonols.</b>	
Myricetin (1, 3, 3', 4', 5'-Pentahydroxyflavonol).....	Magenta.
Quercetin (1, 3, 3', 4'-Tetrahydroxyflavonol).....	Scarlet red.
Isorhamnetin (1, 3, 4'-Trihydroxy-3'-methoxy-flavonol).....	" "
Morin (1, 3, 2', 4'-Tetrahydroxyflavonol).....	" "
Fisetin (3, 3', 4'-Trihydroxyflavonol).....	Yellowish "
Fukugetin (?).....	" "
Kaempferol (1, 3, 4'-Trihydroxyflavonol).....	Scarlet (more yellowish than quercetin).
<b>Flavonol glucosides.</b>	
Myricitrin.....	Same as myricetin (or more bluish).
Quercitrin.....	Crimson.
Rutin.....	"
<b>Flavones.</b>	
Luteolin (1, 3, 3', 4'-Tetrahydroxyflavone)....	Reddish orange.
Apigenin (1, 3, 4'-Trihydroxyflavone).....	Orange.
Chrysin (1, 3-Dihydroxyflavone).....	Golden yellow.
<b>Flavone glucosides.</b>	
Apiin. ....	Yellowish orange.
Toringin.....	Same as chrysin.

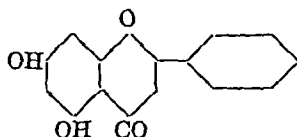
\* The positions of substitutions in the flavone formula are designated as follows:



It is thus seen that the production of red color by reduction in flavones and flavonols is greatly influenced not only by the presence of the hydroxyl group in the benzo-pyrone nucleus, but also by the number and position of the hydroxyl groups in the side ( $\beta$ -) phenyl group. Thus myricetin,



which contains the largest number of auxochrome hydroxyl groups, produces the deepest red color, tending to a bluish hue. On the other hand, a dihydroxyflavone, chrysin.



which contains no hydroxyl group in the  $\beta$ -phenyl group, produces only a golden yellow and no red color by reduction.

Materials were collected at Mt. Shirouma (2,933 meters) in early August, 1915. They included eighty-three species of the alpine flora of the mountains of central Japan known as the Japanese Alps. All were found to contain flavones, a majority showing a very high content in the floral as well as in the vegetative parts. By classifying the plants according to the intensity of the flavone reaction, the following figures are obtained.

Flavone content in the color scale.	Flower.	Shoots and leaves.	Total.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I-II.....	30 (93.8)	45 (57.7)	75 (68.2)
III-IV.....	1 (3.1)	23 (29.5)	24 (21.8)
V.....	1 (3.1)	10 (12.8)	11 (10)
	32	78	110

Among those which are classed in the highest flavone content in the color scale the following



## Leaf.

<i>Alnus viridis</i> var. <i>sibirica</i> .....	I Q <sup>10</sup>
<i>Astragalus membranaceus</i> var. <i>obtus</i> ....	++ I Q
<i>Hedysarum esculentum</i> .....	I Q
<i>Polygonum bistorta</i> .....	I Q
<i>Rhododendron brachycarpum</i> .....	I Q
“ <i>chrysanthum</i> .....	+I Q
<i>Vaccinium vitis-idaea</i> .....	+I Q
<i>Acer parviflorum</i> .....	II Q
<i>Alchemilla vulgaris</i> .....	I-II Q

## Flower.

<i>Acer spicatum</i> var. <i>ukurunduense</i> .....	++I Q
<i>Aconitum pallidum</i> .....	+I Q
<i>Alsine verna</i> var. <i>borealis</i> .....	I A <sup>11</sup>
<i>Anemone narcissiflora</i> .....	I Q
<i>Cnidium ajanense</i> .....	++I Q
<i>Dryas octopetala</i> .....	++I Q
<i>Geum anemonoides</i> .....	++I Q
<i>Hedysarum esculentum</i> .....	++I Q
<i>Sorbus aucuparia</i> .....	+I Q

Owing to the fact that the alpine flowers are rich in flavone, the reaction to ammonia is extraordinarily sensitive. One of the writers observed on Mt. Shirouma that a sprinkle of only a few cc. of ammonia instantly changed every one of the white flowers of *Geum anemonoides* which were within a circle of a half meter to an intense yellow color which remained unchanged for 2 or 3 days.

The leaves of a pine species (*Pinus pumila*) which grows in a high altitude (Mt. Shirouma, at 2,700 meters) are highly rich in flavone, but those of the allied species grown in a lower altitude (Tokyo Botanical Garden) contain it only in a small amount. An analogous case is observed in the leaf of the cowberry (*Vaccinium vitis-idaea*) and others.

The investigation was extended to the plants of the Swiss Alps (St. Gothard, Pilatus, and Rigi), the material being obtained from the herbarium collected by Professor Asahina in 1909 to 1911. All of twenty-two species examined give positive results. It is of interest to note that the silvery hair of a well

<sup>10</sup> Q: The reduction color corresponds to that of pure quercetin.

<sup>11</sup> A: The reduction color corresponds to that of pure apigenin.

known alpine plant, *Leontopodium alpinum* (*Edelweiss*) is very rich in flavone. An exposure to ammoniacal vapor produces at once a deep yellow color and the alcoholic extract gives a beautiful reduction color. These reactions can be observed even with material which has been thoroughly dried and kept for many years. The biological significance of the pubescence of the plant, formally interpreted, is that it serves to protect the plant from excessive transpiration, but considering the evidence at hand, it seems more probable to regard it as the chemical ray filter of the intense insolation in the alpine region.

In closing, a word may be given to an experiment in acclimatization. Kerner (1876)<sup>12</sup> sowed the seed of *Satureja hortensis* and *Linum usitatissimum* in his garden, near Kuppe des Blasers (2,195 meters) in Tirol. The seedlings of the former grew vigorously, but those of the latter were killed shortly after the germination by the intense sunlight of the high altitude. His interpretation of the phenomenon was that the anthocyanin pigment produced by the former protects the chlorophyll from the excessive insolation, so that the young plant is able to develop. We have examined a green specimen of *Satureja* from Lugano and found it rich in flavone. Kerner's observation can now be more adequately explained by our theory.

#### *The Relation of Autumnal Color and the Flavone Content in Foliage.*

In this part the relation of the autumnal coloration and the flavone content in the leaves of deciduous trees of mountain forests is studied. The material was collected in September, just before the leaves became tinged, in the mountain region of Nikko<sup>13</sup> where the autumnal coloring is very marked. The result of the investigation shows that flavones are already present in a sufficient amount in the green leaves (occurring mostly in the epidermal to the palisade cells) to produce anthocyanin in a later season of the year. The formation of anthocyanin is, therefore, a simple biochemical reaction; *i.e.*, a reduction of already existing flavones, initiated by the physiological changes in the living tissue at the end of the growing season.

<sup>12</sup> Kerner, A. J. v., *Pflanzenleben*, Leipsic, 1896, i, 379.

<sup>13</sup> The altitude of the region varies from 650 meters (Nikko Botanical Garden) to 1,543 meters (Yumoto).

The leaves of a plant of high flavone content, however, do not necessarily show the autumnal color. In such cases, flavones disappear from the falling leaves which give no indication of anthocyanin formation.<sup>14</sup>

The plants examined number forty-two species, including genus *Acer*, *Betula*, *Enkianthus*, *Fagus*, *Quercus*, *Prunus*, *Sorbus*, *Viburnum*.

### *The Flavone Content of Tropical Plants.*

The tropical flora form another group of plants which grow in an intensity of light as great as that of high altitude. We have examined a large number of tropical and subtropical plants which are cultivated or grown in the wilds of Formosa.<sup>15</sup> Material was collected in the latter part of January, 1916, the larger part being studied in a fresh condition at the chemical laboratory of the Research Institute of the Formosan Government at Taihoku. The remainder was dried and brought to Tokyo where further work was done.

We have also examined the flavone content of the insular flora of Micronesia (including the Island Yap, Jaliut, Saipan, and Truk)<sup>16</sup> with the herbarium material which was collected by Dr. Koidzumi in the winter of 1914 to 1915. The result of the investigation shows the following:

Flavone content in the scale of reduction color.	Number of plants			
	From Formosa			From Micronesia
	Northern	Southern.	Total	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I-II.....	48 (34.3)	25 (42.4)	73 (36.7)	48 (60.0)
III-IV.....	44 (31.4)	16 (27.1)	60 (30.1)	16 (20.0)
V-VI.....	33 (23.6)	12 (20.3)	45 (22.6)	13 (16.3)
Below.....	15 (10.7)	6 (10.2)	21 (10.6)	3 (3.7)
	140	59	199	80

<sup>14</sup> The decomposition products of flavones are very likely to be carried off from the leaves and stored up elsewhere, probably being converted into tannin.

<sup>15</sup> The island of Formosa and the Pescadores Islands lie between 21° 45' and 25° 38' N., and between 119° 8' and 122° E. The northern half of the island is subtropical, the southern more nearly tropical.

<sup>16</sup> The Micronesian Islands lie between the equator and 20° N. and between 130° and 173° E. The climate is typically insular and tropical.

By classifying the above figures according to the part of the plant in which the flavone content is determined, the following table is obtained.

Flavone content.	Part examined.					
	Leaves.	Stem.	Flower.	Bark.	Wood.	Total.
I.....	46	1	1	2	1	51
II.....	65		3	1		69
III.....	35			2		37
IV.....	30	3	1	2		36
V.....	27	2		1	1	31
VI.....	29		1	1		31
Below.....	17	2		3	2	24
	249	8	6	12	4	279

The total number of plants examined embraces 242 species, extending to 64 families and 190 genera. They include mosses, ferns, grasses, conifers, palms, and many species of angiosperms, and from the ecological standpoint they include epiphyte, parasite, xerophyte, hydrophyte, halophyte, carnivorous plants, and others. Examples of the tropical plants which are of high flavone content are:

Palms with few exceptions, *i.e.*,

<i>Caryota urens</i> .....	I A
<i>Cocos nucifera</i> .....	II A
<i>Corypha australis</i> .....	II A
<i>Elæis guinensis</i> .....	II A
<i>Phoenix hanceana</i> .....	I A

Most of the legumes, such as,

<i>Adenanthera pavonina</i> .....	I Q
<i>Cæsalpinia bonducella</i> .....	I A
<i>Tamarindus indica</i> .....	+II A
<i>Pithecolobium dulce</i> .....	I Q

The mangroves,

<i>Bruguiera cylindrica</i> .....	I Q
<i>Rhizophora mucronata</i> .....	II A

Some of the tropical cultivated plants, such as,

<i>Erythroxylum coca</i> (coca tree).....	I Q
<i>Coffea arabica</i> (coffee tree).....	II A
<i>Bixa orellana</i> .....	II Q
<i>Cinnamomum camphora</i> (camphor tree).....	III Q
<i>Carica papaya</i> .....	II A

<i>Ricinus communis</i> (castor oil plant)...	II Q
<i>Terminalia chebula</i> .....	I Q
<i>Nepenthes phyllamphora</i> .....	++I Q
<i>Diospyros criantha</i> .....	I A
<i>Pistacia formosana</i> .....	+I Q

Palms and mangroves constitute perhaps the most typical form of tropical vegetation which thrives well in the habitat of extreme sunshine. As already mentioned, the leaves of these two groups of plants, the bark of the aerial root (*Bruguiera cylindrica*) and the respiratory root (*Avicennia officinalis*) of the latter show a very high flavone content, while *Didymosperma Engleri* (a palm), *Asplenium nidus* (an epiphytic fern), *Alocasia cucullata* and others which are found in shady woodlands, show a very low flavone content. In another group of plants, a similar case is found, in spite of the fact that they are grown in a very sunny habitat. Some of the fiber-yielding plants (*Agave vivipara*, *Fourcroya gigantea* etc.) and others may be mentioned as examples. Scarcity of flavone in these plants may be due to the fact that their epidermis is covered by a well developed cuticular layer<sup>17</sup> which may serve, somewhat in a similar manner as the dissolved flavone in the cell sap of the epidermis, to extenuate the action of ultra-violet rays.

A type of leaf which possesses a thick cuticula in the epidermis, such as *Ficus elastica* (India rubber), *Artocarpus indica* (Bread tree), etc., is usually low in flavone content.

It is of some interest to note that a species of the tropical mistletoe (*Viscum liquidambericolum*) is very rich in flavone. The reduction color produced by the extract is a deep magenta red, while a common mistletoe (*Viscum album*), collected at Tokyo in winter, contains almost no flavone.

It is observed in many of the tropical plants that when they are cultivated continuously throughout the year in the greenhouse in the temperate zone, their flavone content is considerably diminished. In this connection, it is almost superfluous to mention that the glass covering of the greenhouse almost completely absorbs the ultra-violet rays of the sunlight.

The biological significance of the dissolved flavone derivatives in the cell sap of epidermis in tropical plants may be considered

<sup>17</sup> Kluywer, A. J., *Sitzungsber. Akad. Wiss. Wien*, 1911.

as somewhat analogous to that of the pigment in the skin of the inhabitants of the tropics.

In conclusion, it must be stated that the flavones are the primary product of plant metabolism. They are formed in the germinating seed, growing shoot, etc., upon exposure to daylight. The dependence of the flavone formation upon sunlight can be illustrated in the case of the onion bulb. The inner fleshy leaf scales of the onion bulb, when kept in the dark, contain only a trace of flavone, but on exposing them to strong illumination for a few days, a considerable amount of flavone is formed in the peripheral tissue layers.

#### SUMMARY.

1. A general occurrence of flavone derivatives in the plant kingdom is established.

2. The occurrence of flavone derivatives in plants is almost exclusively limited to the epidermis and the peripheral parenchymatous layer of the aerial parts with few exceptions on record, in which a considerable amount is also found in the bark and the wood, such as in *Myrica rubra*, *Quercus tinctoria*, *Morus tinctoria*, etc.

3. The amount of flavone derivatives contained in the plant tissue can be measured colorimetrically by means of comparing the intensity of reduction color (anthocyanin) produced by an aqueous or alcoholic extract from the fresh as well as from preserved herbarium (dried) material.

4. It is assumed that flavone derivatives dissolved in the cell sap possess an important physiological significance in absorbing ultra-violet rays of the sunlight, by which the living protoplasm and its biochemical agencies are protected from the injurious action of the rays.

5. The plausibility of the above assumption is justified by the results of extensive studies made on plants from alpine and tropical regions where the intensity of the rays considered is high. The plants which are grown in strong insolation are always rich in flavones, except those which are fully protected from the action of the excessive illumination by some means of a morphological and anatomical nature.

6. The green leaves of deciduous trees, which produce anthocyanin pigment in autumn, contain a considerable amount of flavones. The production of autumnal color (anthocyanin) is due to the biochemical change; *i.e.*, the reduction of already existing flavones in the leaf, initiated by the physiological condition at the end of the growing season of the year, without having special ecological significance.

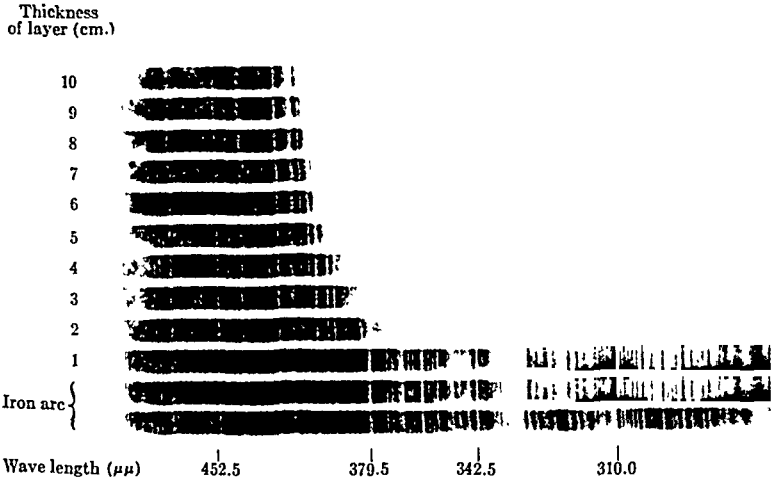


FIG. 1.  
Quercitrin 1/10,000 mol.

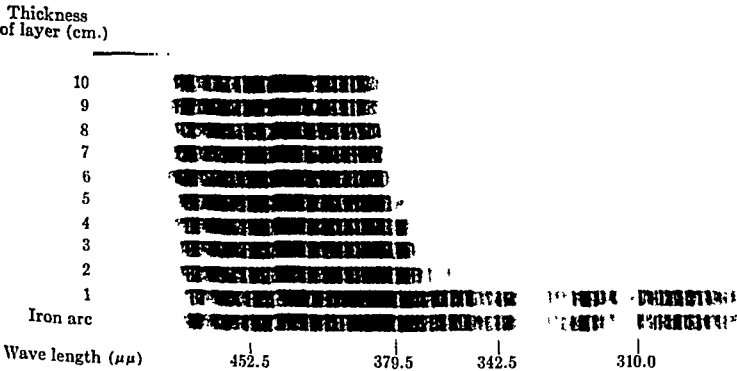


FIG. 2.  
Apigenin 1/10,000 mol.





## PROTEIN COPPER COMPOUNDS.\*

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The use of copper salts for precipitating proteins which can not otherwise be easily brought to separate from aqueous solutions was first employed by Ritthausen,<sup>1</sup> who also attempted to determine the composition of the copper compounds prepared from several vegetable proteins. He later developed a method for analyzing milk in which the proteins were separated as copper compounds. During subsequent years several attempts were made by others to obtain definite compounds of proteins with copper and other metals, but as the methods employed were wholly different from those of the experiments here described they need not be referred to further.

Since the nutritive value of the proteins of the various animal and vegetable products that are used as food for men and animals is now known to depend so largely on the relative proportions of the amino-acids yielded by them on hydrolysis, and since a combination of these food products which will yield the optimal proportion of the essential amino-acids should prove to be the most economical in use, it has become important to know the amino-acid make-up of the total protein contained in the more widely used food products. Attempts to gain such information have been made by subjecting the products of hydrolysis of the entire food products to analysis by the Van Slyke method, but the results in most cases have been unsatisfactory,<sup>2</sup> chiefly

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>1</sup> Ritthausen, H., *J. prakt. Chem.*, 1872, v, 215. Ritthausen, H., and Pott, R., *ibid.*, 1873, vii, 361. Ritthausen, *ibid.*, 1877, xv, 329.

<sup>2</sup> Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762. Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii, 477.

because such a large proportion of the products of hydrolysis of non-protein substances are also present, especially among those obtained from vegetable foods.

Inasmuch as nearly all of the nitrogen can be extracted from most food products by means of dilute alkalis, it seemed possible that by applying Ritthausen's method practically all of the protein thus extracted could be precipitated as copper compounds, and that these precipitates would afford a more suitable material for a study of their amino-acid constituents than does the entire food product. Ritthausen added solutions of copper sulfate and potassium hydroxide alternately to the solution containing the protein until a sample of the precipitate produced failed to dissolve completely in an excess of the alkali. The deep blue precipitate was washed by decantation, dissolved in a very slight excess of alkali, the solution filtered, and the protein copper compound reprecipitated by neutralizing with an acid. Ritthausen regarded such copper compounds of most proteins as wholly insoluble in water, but stated that some of those formed by proteins which were themselves soluble in water were decomposed on washing, a part of the protein going again into solution. He also concluded that the protein entered into combination with the copper without suffering any change, and as proof of this offered analyses of numerous preparations which, when calculated ash-free, agreed closely in ultimate composition with the original preparation from which they were derived. He especially emphasized the fact that no loss of nitrogen occurred, even when the protein had been dissolved for many hours in dilute alkali.

From Ritthausen's papers no definite conclusions can be drawn in respect to many points which should be clearly established if this process is to be used for isolating proteins from extracts of animal or vegetable origin, or for separating them from other nitrogenous substances. It is probably for this reason that Stutzer's method<sup>3</sup> has been generally used for this purpose, since in describing it he directed attention especially to the completeness of the precipitation of the protein and the failure of non-protein substances to be precipitated also, even though these in the pure state form quite insoluble copper compounds.

<sup>3</sup> Stutzer, A., *J. Landw.*, 1881, xxix, 473; also *Landw. Versuchsstat.*, 1882, xxvii, 323.

For the purpose of obtaining relatively large quantities of copper protein compounds, containing all of the protein present in an extract, it seemed that such could be produced better by liberating the copper hydroxide in the presence of the protein according to Ritthausen's method rather than by Stutzer's method, according to which the copper hydroxide is suspended in the solution and the reaction completed by heating on a water bath.

#### EXPERIMENTAL.

As a first step in determining the reaction between copper sulfate and an alkaline solution of a protein, we prepared decinormal solutions of sodium hydroxide and of copper sulfate with freshly boiled water and titrated them against one another. To 40 cc. of boiled water 10 cc. of 0.1 N  $\text{CuSO}_4$  were added and then measured quantities of the alkali. With 0.6 cc. a permanent precipitate began to form which increased in amount until 7.3 cc. had been added. The solution had then become neutral to litmus, and when filtered was shown by hydrogen sulfide to contain only a trace of copper. With 7.5 cc. the reaction was still neutral and no copper was dissolved. An alkaline reaction was not shown until 9.5 cc. had been added. It is evident that an insoluble basic salt is thus formed which reacts promptly with the alkali until a nearly complete normal reaction is attained. The formation of a basic salt is also indicated by the color of the precipitate produced by less than 7.3 cc. of the alkali, for this was very much paler than the deep blue precipitate formed when 10 cc. of the alkali were added.

When the mixture was made in the reversed order evidence of the formation of a basic salt was likewise obtained, for when 9 cc. of the copper solution were added to 10 cc. of the soda solution the reaction was strongly alkaline; with 9.5 cc. it was distinctly alkaline, but with 10 cc. it was neutral to litmus and contained no dissolved copper; with 12 cc. it was likewise neutral to litmus, no copper being dissolved. With 13 cc. the reaction was still neutral, and only a slight trace of copper soluble. We thus find that 7.4 cc. of the soda solution precipitate all of the copper from 10 cc. of the copper sulfate, whereas 13 cc. of the copper solution can be added to 10 cc. of the alkali before evidence of copper appears in the filtrate. The basic copper salt is formed to about the same extent under the two conditions of precipitation for 7.4 : 10 : : 10 : 13.5.

A solution of edestin was made by suspending 2 gm. of a very pure preparation of air-dry edestin in 100 cc. of freshly boiled water and then adding 100 cc. of 0.1 N  $\text{NaOH}$  solution. Nitrogen was determined in 10 cc., 0.0166 gm. being found, equal to 0.0893 gm. of edestin ( $N \times 5.38$ ). This solution of edestin contained

alkali equal to 0.05 N NaOH; hence 10 cc. would require 5 cc. of the 0.1 N  $\text{CuSO}_4$  for a complete reaction. The freshly prepared alkaline edestin solution required 4.9 cc. of 0.1 N HCl for neutralization to phenolphthalein. The acidity of the dissolved edestin was therefore equal to 0.1 cc. of 0.1 N HCl.

*Titration of the Alkaline Edestin Solution with Copper Sulfate Solution.*  
 40 Cc.  $\text{H}_2\text{O}$  + 10 Cc. 0.05 N NaOH + Edestin.

0.1 N $\text{CuSO}_4$ . cc.	Reaction.	Reaction to litmus.
+0.1	Biuret color begins.	Alkaline.
+3.9	Violet-blue.	"
+4.4	Deep blue. Trace of ppt. Solution clear up to this point.	"
+4.6	More ppt.	Slightly alkaline.
+4.8	" "	"
+4.9	" "	"
+5.0	Precipitation not quite complete, ppt. wholly soluble in excess NaOH.	Neutral.
+5.1	Precipitation complete. In solution Cu none, N 0.0002 gm.	"
+5.5	" " " " " 0.0002 "	"
+5.8	" " " " " 0.0002 "	"
+6.0	" " " " " 0.0002 "	"
+6.5	" " " trace " 0.0002 "	"
+7.0	" " " slight trace, N 0.0002 gm.	
+7.5	" " " more, N 0.0002 gm.	
+8.0	" " " " " 0.0002 "	

After standing for 14 days, at about  $20^\circ$ , tightly stoppered and protected by toluene, the solution was giving off ammonia. The edestin was precipitated with 5.4 cc. 0.1 N  $\text{CuSO}_4$ . The filtrate from the edestin copper contained a trace of copper and 0.0020 gm. of nitrogen. The original solution contained 0.0166 gm. of nitrogen in 10 cc.; hence the alkali had split 12 per cent of the nitrogen from the edestin during 14 days. When completely hydrolyzed by boiling with acids, edestin yields 10 per cent of its nitrogen as ammonia. It thus appears that when edestin loses amide nitrogen a product remains which is as completely precipitated by copper salts as is the unaltered edestin.

The above data show that when 5.1 cc. of 0.1 N  $\text{CuSO}_4$  had been added the solution contained only 0.0002 gm. of nitrogen; hence 98.8 per cent of all of the nitrogen had been precipitated. It will be noted that no precipitate began to appear until 4.4 cc. of 0.1 N  $\text{CuSO}_4$  had been added, and that the biuret color, which with 3.9 cc. had assumed a strong blue tint, with 4.4 cc. changed to a deep blue, similar to that of an ammoniacal copper solution, but much less intense. Beyond this point the precipitate increased in amount as further additions of the copper solution were made, until with 0.1 cc. above the quantity required for a complete reaction all of the edestin, as well as the copper, had been precipitated. It thus appears that the edestin copper is as good as insoluble in water, for the trace of nitrogen in the filtrate is doubtless ammonia split from the edestin by the alkali (see p. 112).

It is to be noted further that an excess of 0.1 N  $\text{CuSO}_4$ , of even 3.0 cc. above that required for a complete reaction, did not dissolve any of the edestin copper, for after removing the precipitate from the solution to which 8 cc. of 0.1 N  $\text{CuSO}_4$  had been added no more nitrogen was found than in that containing 5.1 cc. A basic salt is apparently formed to about the same extent as when the copper sulfate is added to the sodium hydroxide solution; namely, in the proportion of 10:13 (p. 111). This formation of a basic salt was noted by Ritthausen.<sup>4</sup> That no copper hydroxide was precipitated with the edestin copper when 5 cc. of 0.1 N  $\text{CuSO}_4$  were added, is shown by the fact that the precipitate was completely soluble in a slight excess of 0.1 N  $\text{NaOH}$ .

In order to learn how edestin, when dissolved in an acid solution, behaves towards copper sulfate the following experiments were tried. 2 gm. of air-dry edestin were suspended in 100 cc. of boiled water and dissolved by adding 100 cc. of 0.1 N  $\text{HCl}$  solution. This contained 0.0166 gm. of nitrogen in 10 cc. or 0.0893 gm. of edestin ( $0.0166 \times 5.38$ ).

<sup>4</sup> Ritthausen, *J. prakt. Chem.*, 1877, xv, 332.

*Titration of the Acid Edestin Solution Containing Copper Sulfate with Sodium Hydroxide Solution.*

*40 Cc. H<sub>2</sub>O + 10 Cc. 0.05 N HCl + Edestin + 10 Cc. 0.1 N CuSO<sub>4</sub>.*

0.1 N NaOH. cc.	Reaction.	In solution.		Reaction to litmus.
		Cu	N gm.	
+ 1	No ppt.		0.0166	Acid.
+ 2	" "		0.0166	"
+ 3	" "		0.0166	"
+ 3.8	Trace ppt.			"
+ 4.1	Distinctly more ppt.			"
+ 5.0	Much white ppt.	Much.	0.0006	"
+ 7.5	" pale blue ppt.	"		"
+ 8.5	" " " "	"		"
+ 9.5	" " " "	Some.		"
+10.0	" " " "	"		"
+11.0	" " " "	Less.		Faintly acid.
+12.0	" " " "	Trace.		Neutral.
+13.0	" " " "	None.	None.	"
+13.8	" " " "	"		"
+14.0	" " " "	"		"
+14.2	Much more blue.	Trace.	Trace of edestin.	Faintly alkaline.
+15.2	Deep blue ppt. Distinctly violet solution.			Alkaline.
+15.5	Nearly all dissolved.			
+15.7	Only a trace undissolved, deep blue solution.			
+18.0	"			

On adding 5 or 6 cc. of 10 per cent NaOH a clear, deep blue solution with very slight violet tint resulted.

From the above data we find that no precipitate begins to form until 3.8 cc. of the alkali are added. With 5.0 cc. practically all of the edestin was precipitated, since the filtrate contained only 0.0006 gm. of nitrogen. The precipitate produced by 5 cc. of 0.1 N NaOH was white, and when washed with water and dissolved in excess of alkali gave only a slight biuret reaction which became strong on adding a little CuSO<sub>4</sub>. These facts indicate that this precipitate contains very little copper, but do

not show whether this is combined or adsorbed. On continuing the addition of alkali the solution became neutral to litmus with 12 cc. of 0.1 N NaOH and contained only a trace of copper. That the precipitate contained a basic copper salt is shown by the fact that the reaction remained neutral until 14.2 cc. of the alkali had been added and that the precipitates produced up to this point were completely soluble in an excess of alkali, provided the stated quantities of 0.1 N NaOH had been added rapidly. The precipitate yielded by 14 cc. of 0.1 N NaOH, when dissolved by a minimal amount of alkali, retained its original deep blue color when diluted with five volumes of 1 per cent sodium hydroxide solution, no sign of a violet tint being produced. If the larger quantities of alkali were added slowly some  $\text{Cu}(\text{OH})_2$  at first remained undissolved, probably owing to the fact that a basic salt was formed under these conditions, but on stirring the slight turbidity slowly disappeared. When 14.2 cc. of 0.1 N NaOH had been added the copper edestin compound began to dissolve. This is 0.8 cc. less than should be required for a complete reaction. Practically all of the precipitate dissolved on increasing the addition of 0.1 N NaOH to 15.7 cc., 0.7 cc. more than is required for a complete reaction between the hydrochloric acid and the copper sulfate which the solution contained, or 1.5 cc. more than the quantity added when the precipitate began to dissolve. The very small amount of undissolved substance,  $\text{Cu}(\text{OH})_2(?)$ , was readily soluble when the quantity of alkali was materially increased. A permanent alkaline reaction to litmus was not obtained until 14.2 cc. of 0.1 N NaOH had been added; with phenolphthalein this reaction developed with 13.4 cc. Nearly the same amount of alkali was required when the edestin was not present in the solution, an alkaline reaction to litmus appearing when 14.0 cc. had been added, or practically at the same point as in the presence of edestin.

To determine if possible the relation of the copper to the white precipitate, produced by adding 5 cc. of the alkali, as well as to the blue one formed by the larger quantities, we repeated these experiments. After adding 5 cc. of 0.1 N NaOH the solution was centrifuged and decanted. The white precipitate was ground up with water, separated by centrifuging, and washed on a filter. The washings gave no biuret reaction. The precipitate after



remaining on the paper over night contracted to a much smaller volume and then showed a distinct greenish tint. When ignited this left an ash weighing 0.0012 gm., equal to 1.3 per cent of the edestin. This was largely, if not wholly, copper oxide. To the solution, from which the white precipitate had separated, 0.5 cc. of 0.1 N NaOH was added before a precipitate was produced, which gradually increased in amount with more alkali, and appeared to be copper hydroxide. When 7.1 cc. of 0.1 N NaOH had been added the solution, which was then neutral to litmus, was centrifuged and decanted from the light blue precipitate. The reaction became slightly alkaline on adding 0.1 cc. more alkali and distinctly alkaline with 0.3 cc., no more precipitate being formed and no copper being in the solution. It thus appears that after removing the white precipitate by adding 5 cc. of 0.1 N NaOH, the solution behaves exactly like one containing only copper sulfate.

Since edestin is insoluble in water at a neutral reaction, and the slight acid reaction of the solution after adding 5 cc. of 0.1 N NaOH may be due simply to the dissociation of the small amount of copper sulfate which it contained, it is probable that the white precipitate consisted of edestin, precipitated by neutralizing the hydrochloric acid by which it had been dissolved. The small amount of copper in the precipitate may have been adsorbed, or it may have been chemically combined with the edestin, possibly representing a molecular compound containing a relatively small number of copper atoms.

If the precipitate, which forms on adding 5 cc. of 0.1 N NaOH, is left in the solution during the further addition of alkali, until the acid reaction to litmus disappears, the blue precipitate then formed may consist of a mixture of free edestin and copper hydroxide, or it may be a compound of edestin with copper. That it does not contain *free* edestin is indicated by the fact that on drying in the air a bright blue substance is left which is practically insoluble in a relatively large excess of alkali, and that it contains *no copper hydroxide* is shown by the fact that in the freshly precipitated condition it is readily soluble in a very little dilute alkali. On the other hand, it is possible that a soluble edestin copper compound may be formed *after* adding an excess of alkali. Since none of the preceding experiments, either with the alka-

line or acid solutions, show the maximum amount of copper with which edestin can combine, we tried the following.

*Titration of the Acid Edestin Solution Containing Copper Sulfate with Sodium Hydroxide Solution.*

40 Cc.  $H_2O$  + 10 Cc. 0.05 N  $HCl$  + Edestin + 35 Cc. 0.1 N  $NaOH$ .

0.1 N $CuSO_4$ .	Reaction.	$CuO$ insoluble.
cc.		
+ 9	Clear blue solution.	None.
+10	" " "	"
+11	" " "	"
+12	" " "	"
+14	" " "	"
+15	" " "	Trace.
+16	Turbid " "	0.0140 gm.
+18	" " "	0.0214 "

The 35 cc. of alkali were added as rapidly as possible with constant stirring. With 12 and 14 cc. there was a small amount of substance which did not dissolve immediately, but on stirring for about 20 to 30 minutes the solutions became perfectly clear. After the addition of 15 cc., followed by stirring for 30 minutes and centrifugation, a just visible amount of  $Cu(OH)_2$  remained undissolved. In the solutions containing 16 cc. and 18 cc. of 0.1 N  $CuSO_4$  there was more undissolved copper hydroxide. The amount of dissolved copper in the experiment with 15 cc. of the  $CuSO_4$  was 0.0474 gm.  $Cu$ . If the  $Cu$  was all combined with the 0.0893 gm. of edestin present in the solution the compound would contain nearly 35 per cent of copper. Repetition of these experiments, however, showed that it is impossible to determine accurately the amount of copper thus combined, for the combination is so unstable that uniform results cannot be obtained. All we can say is that edestin, under the above conditions, for a time can hold in solution a quantity of copper of approximately the above order. After a few hours evidence of change is shown by the blue color of the solution giving place to a brown, which may or may not be accompanied by deposition of insoluble matter. In the former case the solution assumes the characteristic color of the biuret reaction, as this is given by edestin, the brown color being caused by the precipitate of

partly dehydrated copper hydroxide. Solutions to which 14 and 16 cc. of 0.1 N  $\text{CuSO}_4$  had been added, and then 35 cc. of 0.1 N  $\text{NaOH}$ , after standing over night in stoppered flasks were centrifuged and the brown precipitates washed on filters, ignited, and weighed as copper oxide. They yielded respectively 0.0396 and 0.0472 gm. of  $\text{CuO}$ . Subtracting these quantities from the total copper oxide in 14 and 16 cc. of 0.1 N  $\text{CuSO}_4$  leaves in the biuret colored solution 0.0161 and 0.0165 gm. of  $\text{CuO}$  equal to 0.0130 gm. of  $\text{Cu}$  combined with the 0.0893 gm. of edestin. These data indicate that the edestin copper compound which gives the biuret color in alkaline solution contains about 12.7 per cent of copper. It is thus evident that the edestin compounds containing quantities of copper near the maximum are very unstable at the room temperature. The biuret-reacting compound, on the other hand, appears to be far more stable, for after standing for several days no evidence of any change was apparent.

*We have already stated that if the edestin in 10 cc. of our solution combines with all of the copper in 15 cc. of 0.1 N  $\text{CuSO}_4$ , the compound would contain 34.67 per cent of copper, corresponding to an atomic ratio of  $\text{Cu} : \text{N}$  as 10 : 15.83, or very nearly 10  $\text{Cu} : 16 \text{ N}$ . Assuming that one  $\text{Cu}$  combines with one  $\text{N}$ , ten out of every sixteen atoms of the nitrogen, or 62.5 per cent of the total nitrogen of the edestin would be thus united. The compound produced by 14 cc., which in nearly all of our many experiments was readily and completely soluble in an excess of alkali, contains 30.7 per cent of copper, the ratio in this case being  $\text{Cu} : \text{N} :: 10 : 16.9$ , equal to 59 per cent of the nitrogen, assuming one  $\text{Cu}$  to be united to one  $\text{N}$ . According to the two experiments described above, the biuret colored compound contained  $\text{Cu} : \text{N} :: 10 : 58$  or  $10 : 57$ . In this case 17.09 and 17.48 per cent of the total nitrogen would be required for union of one atom of nitrogen with one of copper, or a little more than one-third as much as in the unstable, alkali-soluble compound containing the maximum amount of copper.*

*Copper Compounds of Gliadin.*

Since gliadin differs widely from edestin in the relative proportions of various types of nitrogen which it yields by hydrolysis, the experiments just described were repeated with gliadin in order to determine whether or not the differences in the structure of these two proteins would be revealed by differences in their copper compounds.

A solution of gliadin was prepared containing 0.0158 gm. of N, or 0.0902 gm. of gliadin in 10 cc. of 0.05 N NaOH. In the following table the figures given represent the total quantity of copper sulfate solution added for each of the reactions described, and are *not* to be understood to mean amounts in addition to those preceding.

*Titration of an Alkaline Gliadin Solution with Copper Sulfate Solution.*  
*40 Cc. H<sub>2</sub>O + 10 Cc. 0.05 N NaOH + Gliadin.*

0.1 N CuSO <sub>4</sub> .	Reaction.	Reaction to litmus.
cc.		
0.1	Biuret color just visible.	Alkaline.
0.7	" " much stronger.	"
1.8	Color distinctly more blue.	"
2.3	" " " "	"
4.1	" pure blue.	"
4.6	" changed suddenly to much paler blue.	"
4.9	Trace of precipitate.	Slightly alkaline.
5.0	Distinct precipitate.	" "
5.3	Precipitate lighter blue and more voluminous than edestin. All soluble in excess NaOH.	Neutral.
5.4	Precipitation complete, no Cu in solution.	"
5.5	In solution, Cu none, N 0.0006 gm.	"
6.5	" " " trace, " 0.0006 "	"
7.5	" " " more, " 0.0006 "	Acid.
8.5	" " " " " 0.0008 "	"

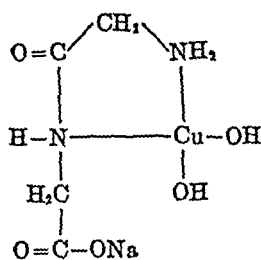
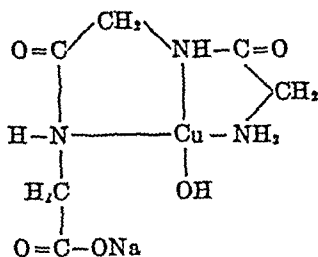
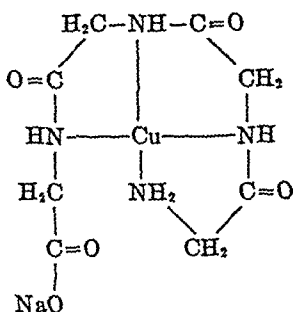
These results agree closely with those obtained with edestin, the neutral point to litmus and the slightly greater amount of nitrogen not precipitated by the copper sulfate being doubtless due to a larger proportion of amide nitrogen split by the alkali from the gliadin than from the edestin. Gliadin yields 25 per cent of its nitrogen as ammonia, when completely hydrolyzed, whereas edestin yields only 10 per cent. The fact that an excess

of nearly 50 per cent of 0.1 N  $\text{CuSO}_4$ , perhaps even more, above that required for a neutral reaction to litmus can be added without dissolving the precipitated gliadin is shown by the nitrogen in solution when 7.5 cc. were added. The slightly greater quantity of nitrogen in solution with 8.5 cc. is within the limits of experimental error. As was the case with edestin a basic salt is formed since no copper was found in solution until 6.5 cc. of 0.1 N  $\text{CuSO}_4$  had been added, or 1.5 cc. more than was required for a complete normal reaction between the sodium hydroxide and the copper sulfate. The color given by gliadin with the smaller quantities of copper sulfate was the pure biuret pink, similar to an alkaline phenolphthalein solution, whereas that given by edestin with corresponding quantities of copper was of a distinctly bluer tint. Both proteins yielded pure blue solutions before precipitation began, which for edestin was with 4.4 cc., for gliadin with 4.9 cc.

The precipitates, thus produced, undoubtedly do not represent definite copper compounds of the protein, for the amount of copper hydroxide available for combination is determined by the amount of sodium hydroxide present in the protein solution, and, as will be shown later, a much larger quantity of copper hydroxide can be rendered soluble in alkalis by the same amount of gliadin than is present in the precipitate produced by 5.3 cc. of 0.1 N  $\text{CuSO}_4$ .

The chemical character of the copper compounds which are formed under the conditions here described has never been established, although it has been the subject of some study. Kober and Sugiura<sup>6</sup> have shown that each molecule of a peptide containing from two to four amino-acids unites with only one atom of copper. For these peptides they represent the combination as follows.

<sup>6</sup> Kober, P. A., and Sugiura, K., *J. Biol. Chem.*, 1912-13, xiii, 1; *Am. Chem. J.*, 1912, xlviii, 383.

Alkaline copper  
glycylglycine.Alkaline copper  
glycylglycylglycine.Alkaline copper  
triglycylglycine.

They state that: "The results on peptides show unmistakably that one molecule of peptide, whatever number of amino-acids it may contain, combines with only one molecule of copper hydroxide." If this statement is strictly true only a few of the peptide-bound nitrogen atoms of the huge protein molecule would be available for union with copper, and accordingly we should expect edestin to be unable to form copper compounds containing approximately 35 per cent of copper. That all of the nitrogen of the R-CO-NH-R groups in edestin can unite with copper hydroxide is indicated by the fact that Van Slyke<sup>6</sup> has shown that edestin yields on complete hydrolysis 62.8 per cent of its nitrogen as amino nitrogen, an amount just sufficient to combine with the maximum amount of copper which we have found to be soluble in an excess of alkali in the presence of edestin. It is improbable that the imino nitrogen of proline reacts with copper, for Osborne and Clapp<sup>7</sup> found that the dipeptide of pro-

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 295.<sup>7</sup> Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907, xviii, 123.

line and phenylalanine, which Fischer and Luniak<sup>8</sup> later showed to be prolylphenylalanine, forms a copper salt with one atom of copper, thus behaving like the dipeptides of the amino-acids. The imino nitrogen of oxyproline and tryptophane will probably behave in the same way, though, as yet, this has not been shown experimentally. According to Van Slyke<sup>6</sup> 25.3 per cent of the edestin nitrogen is imino nitrogen of which about one-half belongs to arginine, or 12.3 per cent of the total nitrogen. The  $\text{NH}_2$  of the guanidine complex of the arginine is here included, for the imino nitrogen as given by Van Slyke is the difference between the total nitrogen and the sum of the ammonia, amino, and melanin nitrogen. The  $\text{NH}_2$  group of the guanidine complex, equal to one-fourth of the arginine nitrogen, or 6.15 per cent of the total nitrogen of the edestin, is thus to be considered. Whether or not, while a constituent of the protein, this is capable of combining with copper is unknown. An arginine copper salt, similar to those formed by the amino-acids has not been described, doubtless because arginine is too strong a base to combine normally with this metal.

Of the remaining 6.85 per cent of the so called "imino nitrogen" a part belongs to proline and a part possibly to oxyproline. These two substances form copper salts which yield copper hydroxide when treated with an excess of alkali and there is no reason to suppose that their  $\text{RC-NH-CR}$  groups will unite with copper as does apparently the  $\text{RC-NH-CO-R}$  group of the peptides and proteins. The same may be said for the imino group in tryptophane, which forms copper salts of the type characteristic of amino-acids and in the dipeptide glycyltryptophane unites with only one copper atom, as shown by Kober and Sugiura. Of the remaining nitrogen, that yielded by hydrolysis as ammonia is probably, though not certainly, present in the protein as amide nitrogen  $\text{R-CONH}_2$ . What part in forming copper compounds may be taken by such nitrogen when a constituent of the protein molecule remains to be demonstrated, but there is no good reason to suppose that this group will unite with the copper, except under such conditions as assumed by Kober and Sugiura for alkaline copper diglycylasparagine. We thus see that little,

<sup>8</sup> Fischer, E., and Luniak, A., *Ber. chem. Ges.*, 1909, xlii, 4752.

if any, imino nitrogen, other than that belonging to the  $R-CO-NH-R$  grouping, remains with which the copper hydroxide can combine, and we are forced to assume that it is with this nitrogen that nearly all of the copper unites. It may be added that histidine forms a copper complex which dissolves in excess of alkali, giving a solution having a color similar to that of the biuret reaction. The proportion of histidine in edestin, however, is too small to affect appreciably the results here under discussion.

On page 117 we stated that the copper edestin compounds which contained the larger proportion of copper and which dissolve in alkali giving deep blue solutions, on standing decompose with separation of copper hydroxide and a change of color to the characteristic violet of the biuret reaction. In the experiments there described the solution contained 0.0893 gm. of edestin and 0.0130 gm. of Cu, equal to 0.1023 gm. of an edestin copper compound, containing 12.71 per cent Cu, and 16.14 per cent of nitrogen, a ratio of  $Cu : N :: 10 : 57.5$ . Since 62.8 per cent of the edestin nitrogen is peptide-bound there would be available for every ten atoms of the copper in this compound thirty-six atoms of such nitrogen out of every hundred, or nearly  $1 Cu : 4 N$  which the experiments of Kober and Sugiura seem to show to be necessary for the formation of a true biuret color. The color of the solutions containing copper in the above ratio is not the "true biuret" color given by tetrapeptides, but the "semibiuret," which is that given by all of the native proteins. It is highly probable that besides the union of four nitrogen atoms coordinately with the copper a little of the latter is also combined in some other way, in consequence of which a blue-violet tint is imparted to the solution.





## CHINESE PRESERVED EGGS—PIDAN.

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This paper gives the results of analysis of a kind of Chinese preserved eggs called pidan. The Chinese and other Oriental peoples preserve eggs, not necessarily to keep them unchanged, but to make various new substances—a process analogous to the production of cheese from milk. The products have been little studied and the literature on the subject is therefore limited. Hanzawa<sup>1</sup> describes a number of Chinese methods of preservation and gives the results of a bacteriological study of pidan. The descriptive part of his article and also a portion of one by Gibbs,<sup>2</sup> referred to below, are given in almost literal translation by Long.<sup>3</sup> Earlier work is that of Svoboda<sup>4</sup> on Oriental preservation in wood ashes, extensively quoted by König<sup>5</sup> and by Kossowicz.<sup>6</sup> In 1908 a paper appeared by Vosseler<sup>7</sup> on egg preservation in the tropics. Gibbs and his coworkers<sup>2</sup> describe a process of preparing eggs used in the Philippines and give analyses of these and fresh ducks' eggs. The United States Department of Agriculture makes brief mention of Oriental eggs in one of its Farmers' Bulletins.<sup>8</sup> A recent number of the National Geographic

<sup>1</sup> Hanzawa, J., *Zentr. Bakt., 2te Abt.*, 1913, xxxvi, 418.

<sup>2</sup> Gibbs, H. D., Agcaoili, F., and Shilling, G. R., *Philippine J. Sc., Section A*, 1912, vii, 390.

<sup>3</sup> Long, A. L., *Pure Products*, 1913, ix, 494.

<sup>4</sup> Svoboda, H., *Chem. Ztg.*, 1902, v, 483.

<sup>5</sup> König, J., *Chem. mensch. Nahr.- u. Genussmittel*, Berlin, 1914, iii, pt. 2, 175.

<sup>6</sup> Kossowicz, A., *Die Zersetzung und Haltbarmachung der Eier*, Wiesbaden, 1913.

<sup>7</sup> Vosseler, J., *Der Pflanzer*, 1908, iv, 129, cited in *Chem. Abstr.*, 1909, iii, 1780.

<sup>8</sup> Langworthy, C. F., *U. S. Dept. Agric., Farmers' Bull.* 128, 1906.

Magazine<sup>9</sup> gives pictures which are probably of the kind of eggs that we have studied.

*Preparation and Physical Properties of Pidan.*

Pidan is made from ducks' eggs and is a factory product. The following description of its preparation was obtained from the manager of a factory. It is similar to the method given by Hanzawa, but more detailed. To an infusion of  $1\frac{1}{2}$  pounds of strong black tea are stirred in successively 9 pounds of lime,  $4\frac{1}{2}$  pounds of common salt, and about 1 bushel of freshly burned wood ashes. This pasty mixture is put away to cool over night. Next day 1,000 ducks' eggs of the best quality are cleaned and one by one carefully and evenly covered with the mixture, and stored away for 5 months. Then they are covered further with rice hulls, and so with a coating fully  $\frac{1}{4}$  inch thick are ready for the market. They improve on further keeping, however, for at first they have a strong taste of lime which gradually disappears. Eggs preserved in lime-water and salt are also said to have a lime-like taste.<sup>8</sup> The eggs are eaten without cooking.

They are very different from fresh eggs. The somewhat darkened shell has numerous dark green dots on the inner membrane. Both the white and yolk are coagulated; the white is brown, more or less like coffee jelly, and the yolk greenish gray with concentric rings of different shades of gray. The yolk gradually loses its peculiar color on exposure. Numerous tyrosine-shaped crystals are found on the side of the white next to the yolk, apparently formed on the vitellin membrane. The taste of the eggs is characteristic and the odor markedly ammoniacal. It may also be noted here that the eggs have no odor of hydrogen sulfide and that no blackening of lead acetate paper could be detected during 15 minutes' treatment of the finely divided yolk and white with acid in closed weighing bottles.

The appearance of the eggs, however, is not wholly unique, considering the alkalinity of the materials used for preservation. Evéquoz and Häussler<sup>10</sup> observed that when water-glass contains too much alkali, the latter diffuses into the egg, making the white into a yellow transparent mass and solidifying the yolk. König quotes Svoboda to the same effect, and states that Oriental eggs kept in wood ashes become stiffened and look like hard-boiled eggs. The changes in pidan formation are

<sup>9</sup> Showalter, W. J., *Nat. Geog. Mag.*, 1916, xxix, 41.

<sup>10</sup> Evéquoz, A., and Häussler, E. P., *Z. Untersuch. Nahr.- u. Genussmittel*, 1913, xxv, 96.

doubtless also bacteriological. Hanzawa isolated five kinds of bacteria from them, inoculated fresh eggs with these bacteria, and then placed them in water-glass for several months. The resulting substance was similar to pidan in color and hardness.

It is interesting to compare pidan with various published descriptions of decomposed eggs. Kossowicz in the first part of his monograph reviews the literature of the subject, chiefly from the bacteriological point of view, but with numerous descriptions of the spoiled eggs. Especially noteworthy are Gayon's observations<sup>11</sup> that while most of his eggs gave off hydrogen sulfide on spoiling, some which decomposed in the absence of air had an odor of phosphine and ammonia. This is the only reference that we have found to the presence of free ammonia in eggs, or at least in sufficient quantity to give a noticeable odor. Numerous descriptions are also given of eggs darkened in color and of pasty or jelly-like consistency, but most of these dark solid eggs smelled of hydrogen sulfide. The formation of greenish spots of mould or bacteria on the inside of the shell is often noted, both in early work and in Kossowicz's, and tyrosine-shaped crystals have been observed<sup>12</sup> on the vitellin membrane. Poppe<sup>13</sup> adopted Schrank's classification of spoiled eggs as mouldy eggs, eggs smelling of hydrogen sulfide (the typical rotten eggs), and eggs recognized by putrid decomposition and a cadaver-like smell—none of which describes pidan. Thus in spite of numerous points of similarity between these variously described decomposed eggs, none of them resemble pidan closely. Comparisons with cold storage eggs are made below.

### *Methods of Analysis.*

Determinations were made of total solids, ether extract, acidity of ether extract, alkalinity of ash, nitrogen as total nitrogen, coagulable, non-coagulable, volatile, and amino nitrogen, and lecithin phosphorus and total phosphorus. The methods used were chiefly those of the Association of Official Agricultural Chemists.<sup>14</sup>

The egg was first weighed whole, the yolk and white were separated and weighed, chopped into small pieces, and dried in air at 45°C.<sup>15</sup> All but a few tenths of 1 per cent of the water or volatile matter of the yolk and a little less of the white was lost during the process. The total water

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<sup>11</sup> 1873. Quoted by Kossowicz.<sup>6</sup>

<sup>12</sup> Wiley, H. W., *U. S. Dept. Agric., Bureau of Chemistry, Bull. 115*, 1908.

<sup>13</sup> Poppe, K., *Arb. k. Gsndhtsamte*, 1910, xxxiv, 186. Schrank, 1894.

<sup>14</sup> Wiley, *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107* (revised), 1908.

<sup>15</sup> Leach, A. E., *Food Inspection and Analysis*, New York, 3rd edition, 1914.

content was determined by further drying in hydrogen at 100°C. after pulverizing the partially dried substance. The ash and the alkalinity of ash were determined on the residue after total drying, as was also the ether extract of the yolk, the extraction being continued for 20 hours in some cases and 8 in others. In the first ether extract determination petroleum ether was used, but as the result was unexpectedly low, anhydrous ether was substituted thereafter, although it gave only slightly higher results. Several eggs were used in duplicating the moisture and fat determinations. For the nitrogen determinations portions of white and yolk, about 3 gm. each, were weighed in closed weighing bottles containing dilute acid, for it had been noted that the egg when opened gave off ammonia or some volatile ammoniacal substance in sufficient quantity to give a marked odor and promptly to turn a piece of red litmus held over it decidedly blue.

The coagulable, non-coagulable, and volatile nitrogen were determined upon the same portion of the egg. This portion was first ground with hydrochloric acid, neutralized with sodium hydroxide, reacidified with acetic acid to a faint but distinct acidity,<sup>16</sup> and then heated to boiling for 3 minutes. If the acidity was too faint, the solution remained milky after heating, and was hard to filter. The clear filtrate was made up to 150 cc. The residue was used for the determination of coagulable nitrogen by the Kjeldahl-Gunning method, and 100 cc. of the filtrate were taken for the non-coagulable nitrogen. The rest of the solution was used for the determination of volatile nitrogen by Folin's method, on the addition of sodium carbonate and aeration for a number of hours into 25 cc. of 0.1 N acid. In another case the filtrate from the coagulable nitrogen was used for the Van Slyke amino nitrogen test.

### *Results of Analysis and Comparison with Other Eggs.*

The following table gives the percentage of the three parts in four Chinese eggs, and, for comparison, in fresh ducks' eggs. The first figures for the fresh eggs are those given by Lührig<sup>17</sup> as the average of nineteen eggs that he worked with, and the second are those of Wood and Merrill.<sup>18</sup>

The loss in weight in opening and weighing the eggs, averaging 0.6 gm., or about 1 per cent of the total, is probably chiefly water, but also a little ammonia. Eggs 1 and 4 were bought at a different place from 2 and 3.

As shown in Table I, our eggs weighed less than most of the

<sup>16</sup> Pennington, M. E., *J. Biol. Chem.*, 1909-10, vii, 109.

<sup>17</sup> Lührig, H., *Z. Untersuch. Nahr.- u. Genussmittel*, 1904, viii, 181.

<sup>18</sup> Quoted by Leach, p. 264, from Wood and Merrill, *Maine Agric. Exp. Station, Bull. 75*, 90.

TABLE I.

	Whole egg.	Shell.	White.	Yolk.	Loss.	Shell.	White.	Yolk.	Loss.
	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent
Pidan 1.....	58.24	8.18	17.79	31.87	0.39	14.1	30.6	54.7	0.6
" 2.....	64.76	9.29	15.13	39.70	0.64	15.0	23.2	60.9	0.9
" 3.....	52.10	7.85	12.09	31.73	0.44	14.4	23.4	61.3	0.9
" 4.....	65.50	8.89	19.92	36.57	0.12	13.6	30.4	55.8	0.2
Fresh 1.....	67.7	7.7	36.0	24.0		11.4	53.2	35.4	
" 2.....	68.1	7.2	36.5	24.4		10.6	53.6	35.8	

published weights of fresh ducks' eggs, and the whites without exception were far lighter. The percentage of the shell and the yolk of pidan are decidedly higher, while the percentage of the white is only about half that of fresh eggs. That some of the water of the white of pidan must have gone over to the yolk during preservation is further definitely shown in the analysis of pidan given in Table II. Here the percentage of water is 54.0 in the yolk and 69.8 in the white, instead of 45.8 and 87.0 for fresh eggs (Table III). There has thus been a marked transfer of water from white to yolk and a marked loss of water from the white to the air. The difference is still more marked when the actual quantities of water in white and yolk are computed for Egg 1 and for fresh eggs: in the whites, 12.4 gm. in pidan, and 32 gm. in fresh eggs; and in the yolks, 17.1 gm. in pidan, and 11 gm. in fresh ones. Greenlee<sup>19</sup> found a similar though much slighter change in hens' eggs kept in cold storage. He reports a change in the moisture of the yolks during 40 days from 47.17 to 50.60 per cent, and in the whites from 87.60 to 84.74 per cent.

In Tables II and IV are brought together the results of the analyses of the yolk and white of pidan, and in Table III, the average composition of fresh ducks' eggs as given by Langworthy.<sup>8</sup> The first two lines of figures for yolks and whites of Table II, except the nitrogen, are all for the same egg (No. 1), the other figures are for other eggs, possibly of different age, and show considerable variation.

<sup>19</sup> Greenlee, A. D., *U. S. Dept. Agric., Bureau of Chemistry, Cir. 83*, 1911; *J. Am. Chem. Soc.*, 1912, xxxiv, 539.

TABLE II.  
*Composition of Pidan.*

	Water.	Ether extract.	Acidity of ether extract, oleic.	Ash.	Alkalinity of ash, NaOH.	Total N.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Yolk.	53.55	21.06	7.0	4.08	1.79	2.33
	53.59	21.00	6.9	4.06	1.74	2.32
	58.25	17.6				2.20
	55.17	21.9				2.22
	49.57	23.7	9.0			2.14
White.	69.56			3.13	1.21	3.21
	70.02			2.93	1.21	3.20
						3.06
						3.08

TABLE III.  
*Composition of Fresh Ducks' Eggs.*

	Water.	Protein.	Fat.	Ash.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
White.....	87.0	11.1	0.03	0.8
Yolk.....	45.8	16.8	36.2	1.2

A number of differences between pidan and fresh eggs are shown in Tables II and III, in addition to the marked water changes. First, pidan is decidedly higher in ash. Compared with other eggs preserved in alkaline solutions, this high percentage is not surprising. König quotes that eggs kept in wood ashes, if somewhat moist, show in 4 months an increase of ash from 1 to 3 per cent. The ash in these eggs has been found as high as 6.5 per cent. The alkalinity of the ash of König's eggs was also found to be high, whether preserved in wood ashes or in too strongly alkaline water-glass,<sup>10</sup> but not so high as in the yolk of pidan.

The ether extract of the yolk is surprisingly low, 21.1 per cent as compared with Langworthy's 36.2 per cent of the yolks of fresh ducks' eggs. The difference is still marked even when the results are calculated on the dry basis, 44 per cent for pidan and 67 per cent for fresh eggs. Also the acidity of the ether extract is high,

8 per cent free acid calculated as oleic acid. This was determined by titrating duplicate extracts of the yolk of several eggs, using Marcusson's<sup>20</sup> two indicator method, phenolphthalein, and alkali blue B 6. In view of Pennington's observations<sup>21</sup> on the occurrence of lipase in hens' eggs, a high acidity is not unexpected. It is possible also that some of the acid from the action of the lipase and the alkali from the preserving material may have formed soaps. These of course would not be extracted by the ether and would give an alkaline ash.

To test this idea of soap formation several of the residues from the ether extraction were ground up with hydrochloric acid, dried, and reextracted with ether. They all gave further substance to the ether which was completely soluble in warm alcohol. It amounted on the average to 3.1 per cent of the original yolk and titrated roughly as 95 per cent oleic acid. This quantity of substance is, of course, not great enough to account for more than part of the loss in ether extract.

Another possible source of loss of ether extract might be decomposition of the phospholipoids.<sup>22</sup> To find whether this had taken place, we determined the total phosphorus of the yolk and the phosphorus in the extract obtained by successive extractions of the dried yolk with anhydrous ether (12 hours), absolute alcohol (12 hours), and again with ether (18 hours). The phosphorus was determined in the usual way as magnesium pyrophosphate after oxidizing the material with sodium carbonate and potassium nitrate. We found 0.77 per cent total and 0.39 per cent lecithin phosphoric anhydride. This is much lower than figures given by Lührig<sup>17</sup> for fresh eggs. According to these the yolk of ducks' eggs contains 1.255 per cent total  $P_2O_5$ , 0.643 per cent "soluble in ether, pure lecithin," and 0.218 per cent "soluble in alcohol, lecithin bound to nuclein," or altogether 0.861 per cent lecithin  $P_2O_5$ . Of course to compare these figures with ours from our abnormally moist yolk, all should be calculated on the dry basis. If this is done the lowness of our figures is even more striking, although the yolk we used contained less water than most of

<sup>20</sup> Marcusson, J., *Z. angew. Chem.*, 1911, xxiv, 1297.

<sup>21</sup> Pennington, M. E., and Robertson, H. C., *U. S. Dept. Agric., Bureau of Chemistry, Cir. 104*, 1912, and Original Communications, *8th Internat. Cong. Appl. Chem.*, 1912, xxvi, 405.

<sup>22</sup> We wish to thank Dr. David Klein for suggesting this.



ours—49.6 per cent—and weighed much less. Lührig's fresh yolks contained 45.0 per cent moisture. This gives us on the dry basis 2.28 per cent total and 1.59 per cent lecithin phosphoric acid for the fresh yolks, as opposed to 1.53 and 0.76 per cent for ours. Both total and lecithin phosphoric acid are low in pidan, by approximately the same amount—0.8 per cent. The actual quantities in the whole yolks are 0.2987 and 0.2050 gm. for the fresh eggs, and 0.169 and 0.086 gm. for pidan. Thus not far from half the lecithin phosphorus has apparently disappeared, has not been transformed into some other phosphorus compound insoluble in alcohol or ether, but is actually gone from the yolk. Of course the data are insufficient for accurate determination of the amount of change, but there can be little doubt that the lowness of the ether extract of pidan is in part due to some decomposition of phospholipoids.

Besides the change of water, ash, and ether extract in pidan, the figures in Tables II and III seem to show increase in the protein of the white ( $N \times 6.25$ ). This increase is apparent only, however, and due merely to decrease in moisture. Recalculation on the dry basis shows loss rather than gain, 65 per cent of the total solids instead of the 85 per cent of the fresh, probably because of actual increase of ash. The yolk protein so calculated shows little difference between pidan and fresh eggs (29 and 31 per cent).

The distribution of the nitrogen of pidan is given in Table IV. It is compared with some of Pennington's<sup>16</sup> figures for fresh hens' eggs in Table V.

It should be remembered that the figures for pidan are for three different eggs. As might be expected, they are not perfectly concordant, but show definite variations from fresh eggs. The average for Pennington's non-coagulable nitrogen is 9.1 per cent of the average for total nitrogen; whereas the corresponding figures for pidan are 16 per cent for the white and 18 per cent for the yolk. The non-coagulable nitrogen is thus decidedly higher in pidan than in fresh hens' eggs. No figures were found for fresh ducks' eggs.

Pennington also determined ammoniacal nitrogen on fresh and old eggs by the Folin aeration method. An increase was found of ammoniacal nitrogen from 0.0012 per cent in well handled eggs 1

TABLE IV.  
*Distribution of Nitrogen in Pidan.*

	Total.	Coagulable.	Non-coagulable.	Volatile.	Amino.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Yolk.	2.33	2.04	0.42	0.06	
	2.32	1.99	0.41	0.07	
	2.20	1.92	0.41	0.06	0.182
	2.22	1.80	0.44		0.179
	2.14	1.79	0.33		0.179
Average.	2.24	1.91	0.40	0.06	0.180
White.	3.21	2.89	0.54	0.07	0.299
	3.20	2.84	0.59	0.08	0.303
	3.06	2.50	0.44	0.10	0.303
	3.08	2.54	0.44	0.12	
Average.	3.14	2.69	0.50	0.09	0.302

TABLE V.  
*Distribution of Nitrogen in Hens' Eggs.*

Total.	Coagulable by heat.	In filtrate from coagulum.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1.65	1.46	0.195
1.68	1.49	0.193
1.70	1.59	0.112
1.71	1.64	0.109
1.68	1.51	0.152
1.67	1.54	0.156

day old, to 0.0030 per cent or more in an egg which was almost a white rot.<sup>23</sup> This author used portions of the whole egg in the determinations, and we used merely the filtrate from the coagulable nitrogen, but even so the ammoniacal nitrogen in pidan is from twenty to forty times as high as that in the oldest and poorest of Pennington's eggs. Higher figures than Pennington's are given by Houghton and Weber.<sup>24</sup> For black rots they give 169.6 mg. of ammonia nitrogen per 100 gm. of moisture and fat-free substance. Our results calculated on the same basis are 240 mg. per 100 gm. of yolk, and 300 mg. per 100 gm. of white. Even

<sup>23</sup> Pennington, U. S. Dept. Agric., Bureau of Chemistry, Cir. 98, 1912. Other similar figures given in Bull. 224, 1916.

<sup>24</sup> Houghton, H. W., and Weber, F. C., Biochem. Bull., 1914, iii, 447.

these figures may be too low, since ammonia was undoubtedly lost in opening the eggs.

The presence of ammonia is especially important in connection with the low phosphorus content. At least some of it may have come from the lecithin. If it had all come from protein other signs of protein decomposition should have been more marked. The increase above the normal of the non-coagulable nitrogen in pidan does not seem great enough to correspond with this excessive ammonia. Some proteolysis has undoubtedly taken place during the preservation, but also some decomposition of the lecithin.

The amino nitrogen was determined by Van Slyke's method<sup>25</sup> on the filtrate from the coagulable protein of both yolk and white of one of the eggs, using 14.6 gm. of yolk and 8.0 gm. of white to obtain 100 cc. of solution. This was exactly neutralized, evaporated on the water bath to one-fourth its volume, and 2 cc. portions were used for the determination in the micro apparatus. It was necessary to shake this reaction mixture for 18 to 20 minutes to get concordant results. The yolk showed 0.18 per cent amino nitrogen, and the white 0.30 per cent. No figures on amino nitrogen of fresh or cold storage eggs have been found in the literature.

#### SUMMARY.

The following changes have been shown to take place during the formation of pidan from fresh 'ducks' eggs. (1) Water in large quantities has been transferred from the white to the yolk, and water has been lost from the white to the outside. (2) The ash and the alkalinity of ash have increased in a way similar to that of other eggs preserved in alkali. (3) The ether extract has decreased and its acidity is high. (4) Both total and lecithin phosphorus have decreased. (5) The non-coagulable nitrogen has increased and also the ammoniacal nitrogen, the latter to an extraordinary degree, and the amino nitrogen is high. From these changes the conclusion is drawn that decomposition of the egg protein and of the phospholipoids has taken place. The production of pidan from the fresh eggs is probably brought about through the agency of the alkali, bacteria, and enzymes.

<sup>25</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275

# THE COMPOSITION, ESPECIALLY THE HYDROGEN ION CONCENTRATION, OF SEA WATER IN RELATION TO MARINE ORGANISMS.

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Since the extensive analyses of Forchhammer and Dittmar, the relative concentration of the chief salts in sea water has been accurately known, the differences in the results of different investigators only slightly exceeding the differences in the values of the atomic weights that they used. Omitting regions affected by land drainage and the melting of the polar ice caps, ocean surface salinity (gm. of total salts per kilo) varies only from about 33 to 38, the most dilute region being the North Pacific. The known ocean currents affect only the surface layers, but there is probably a drift of the bottom water chiefly from the South and less from the North Polar regions toward the equator, which could take place at a depth of 4,000 meters without obstruction. The evidence for this is the fact that the depths of the oceans have a remarkably uniform salinity of nearly 35 and temperature of about  $2^{\circ}$  which is much above the temperature of maximum density, and are fairly well oxygenated. It seems safe to infer, therefore, that all surface water has comparatively recently come from the depths under the equator.

Dittmar showed that there is proportionately 0.44 per cent more  $\text{CaCO}_3$  at the bottom than at the surface, due to the solution of pelagic shells by means of the  $\text{CO}_2$  from organic decomposition. It is evident, therefore, that the  $\text{CaCO}_3$  brought from the depths and from the land is precipitated in surface water, especially in the tropics. Drew supposes that this is not done entirely by the formation of shells and skeletons, but also by the action of denitrifying bacteria which increases the alkalinity of the water by removal of nitrates until the solubility product for  $\text{CaCO}_3$  is exceeded. Palitzsch has shown that the alkalinity of the sea varies inversely with the depth, but this is probably largely the result of changes in  $\text{CO}_2$  content due to the synthesis of organic matter at the surface and its decomposition in the depths, and less to the changes in nitrates, especially since nitrates and ammonia vary in the same direction. The effect of denitrifying bacteria is probably greatest where the sea is diluted with fresh water. I

found that  $\text{CaCO}_3$  began to precipitate on the glass when the  $P_H (= -\log H^+ \text{ concentration})$  was kept at 8.26, although the addition of  $\text{CaCl}_2$  causes no precipitation.

In selecting a place to study sea water, the coast laboratories are to be avoided, owing to the drainage of the land. Therefore it was decided to go to Tortugas, Florida, where the water may be considered ocean water since it is a deep blue with an average salinity of 36 (35.23 to 36.09) (Dole) and is remarkably free from plankton or sediment. The scarcity of life is probably due to the lack of fixed nitrogen as I found 0.0085 mg. of  $\text{NH}_3$  in a liter and hardly a trace of nitrates in 2 liters. Since Tortugas is about 500 miles from the mouth of the nearest large river, the accession of fixed nitrogen from the land is negligible, and its marine flora is dependent on that coming up from the depths or down with the rain, which escapes the action of the denitrifying bacteria.

The temperature of the Tortugas laboratory (which is practically in the sea) showed a diurnal rhythm of about  $25\text{--}31^\circ$ , and a general drift of about  $2^\circ$  during the season. Most of the experiments were done at a temperature of  $29.75\text{--}30.25^\circ$ . The temperature of the sea was not taken regularly, but it was always near  $29^\circ$ .

The special Leeds and Northrup potentiometer (reading to 0.5 millivolt), Weston cell (of the unsaturated type), and weights (used in making the solutions) had just been standardized by the United States Bureau of Standards. The calomel electrodes were made as follows: A round-bottomed 100 cc. cylinder, with a side neck attached near the top and bending down to the bottom, was supplied with a large platinum electrode fused in the bottom. Redistilled mercury, purified by shaking in a shaking machine for 30 minutes with each of several changes of 3 per cent nitric acid, was dissolved in nitric acid, and this mercurio-nitrate was used in making the mercury by electrolysis. The electrode was filled with the mercurio-nitrate and the side neck dipped into a vessel of mercurio-nitrate containing a platinum anode. A mercury anode was tried and discarded since I could not find a method of preventing the formation of a less soluble mercury salt on its surface. As much current was passed, through a resistance, from the 110 volt direct current circuit as was possible without undue heating of the side neck, until the platinum was plated over its whole surface with a thick layer of mercury, and mercury commenced to drop off of it in considerable amount. The KCl was recrystallized by solution in hot distilled water in fused quartz and cooling (and throwing away the mother liquor) five times, and dried 3 months over  $\text{CaCl}_2$ . The solution was made as needed by weighing out 7.456 gm. and dissolving it in

distilled water in a liter flask. A fresh portion of mercurio-nitrate was purified by electrolysis, redissolved, and precipitated with Baker's analyzed HCl, and the precipitated calomel washed by decantation several times with distilled water and many times with the 0.1 N KCl, always being in contact with excess Hg which was finally shaken to form a gray mixture with the calomel, and washed into the electrode with KCl solution until the platinum was deeply covered. The end of the side neck was closed with an ungreased ground glass cap and passed through the rubber stopper of a cylinder filled with 0.1 N KCl. The electrode was filled with 0.1 N KCl and closed by fusing the glass in a flame. The second cylinder was connected by means of a syphon closed with an ungreased ground glass cap to a third cylinder filled with a saturated solution of KCl. The electrode vessel and part of the side neck were painted black to prevent the reduction of the calomel by light.

The only course left for change of this electrode is change in average size of grain of calomel, due to the greater solubility of the smaller grains, but it seems to be a general opinion that such a process is very slow. The electrodes measured zero against one another when first made up and when they were periodically tested later. They gave the expected E. M. F. against electrodes of several other types. Since I aim at an absolute accuracy of 1 millivolt ( $=0.016 P_H$ ), their drift of potential during the season cannot be considered serious.

The combined tonometer and hydrogen electrode and the improved hydrogen electrode previously described (McClendon and Magoon) were used, with the exception that the latter was made with the large compartment about 11.5 cc., owing to the fact that the buffer value of sea water is less than that of blood. After passing the hydrogen bubble over into the 1.5 cc. compartment the end cock (d) was opened before closing the middle cock (c) in order to restore the pressure that had been reduced by the solution of some hydrogen. Several forms of titration hydrogen electrodes were tried but the one shown in Fig. 1 is to be recommended, owing to the absence of rubber bulb, piston, or mercury funnel to suck up the liquid into the electrode. It is simply immersed in the liquid far enough so that when the cock is properly turned the liquid enters the electrode and drives some of the hydrogen out through the trap T. It is likewise preferable to other non-sucking titration electrodes in being more protected from diffusion of  $O_2$  from the fluid outside.

The hydrogen electrode proper in each of these instruments was made of a gold disc (welded to platinum wire) cleaned with a saturated solution of potassium bichromate in concentrated sul-

furic acid, washed, and platinized with 2 per cent platinum chloride containing a trace of basic lead acetate. It was not cleaned by electrolyzing  $\text{H}_2\text{SO}_4$ , since it was never used as anode and the possibility of the formation of  $\text{Cl}_2$  was excluded. It was replatinized a few seconds after each dozen determinations. Since this process would finally make the platinum black too thick, the life of the electrode may be prolonged by the use of palladium, which can be dissolved off (Clark and Lubs; Ostwald and Luther).

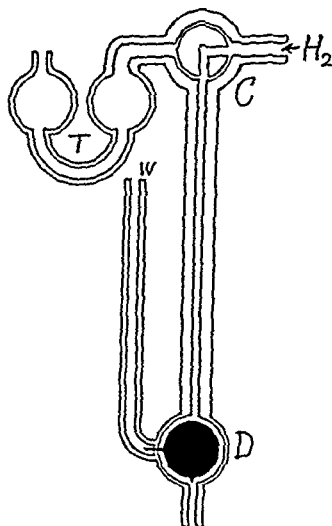


FIG. 1. Hydrogen electrode for titration. Insert a drop of Hg and a copper wire in W. The bulb, D, is immersed in the fluid in a beaker and hydrogen is bubbled through it. The cock, C, is turned so that the bulb fills (by gravity) with fluid which drives hydrogen out at T. Hydrogen is bubbled through again and the fluid allowed to rise until it just touches the platinized gold disc (black), when the reading is taken.

The hydrogen was generated from zinc and  $\text{H}_2\text{SO}_4$  and washed with (1) alkaline permanganate, (2)  $\text{HgCl}_2$ , and (3) alkaline pyrogallol, in all glass, stopperless wash bottles. The  $\text{CO}_2$  was generated from marble and HCl and washed with  $\text{NaHCO}_3$  solution.

Owing to the high electrical resistance in the circuit caused by the 0.1 N KCl in the two ground joints in the calomel electrode (which were always closed) and the sea water in the ungreased stopcock of the hydrogen electrode, which was also closed, the

ordinary capillary electrometer was not sufficiently sensitive to read down to 0.5 millivolt. The capillary tube of the electrometer was drawn out in the flame to about 0.01 mm. bore and until the wall was thin enough (even after pasting a cover-glass on it with Canada balsam) to admit the use of an 8 mm. objective and very high ocular in the reading microscope. By bending back the bulb of the capillary electrometer it was made to fit on the stage of a microscope tilted back and held by rubber bands. In order to prevent shaking, the table legs were passed through the floor into the ground without touching the floor. The use of the capillary electrometer saves much time.

The millivolt readings after correction for  $H_2$  density, according to Clark and Lubs, were converted into  $P_H$  values by means of the conversion table previously published (McClendon, 1916) which was extended sufficiently for the purpose. This change makes all the values in the table about 1 millivolt too low, and therefore 1 millivolt was arbitrarily subtracted from each of Clark and Lubs' corrections. Clark and Lubs find a different theoretical value for the E. M. F. of the normal hydrogen electrode against the 0.1 N calomel electrode from that used in making the conversion table. Since much of the older work was done with HCl, and since Ellis has shown that its curves for dissociation calculated (1) from electrode potential and (2) conductance ratio are different even at considerable dilution, the discrepancy may be due to the use of the conductance ratio to denote the dissociation of HCl at dilutions at which it differs from that calculated from electrode potential.

Since my solutions were near neutrality and the reading was taken immediately after making junction with saturated KCl solution, I do not think that the diffusion potential vitiates my results.

For preliminary tests a series of "nonsol" glass tubes (1 cm. bore) filled with Sørensen's phosphate mixtures plus phenolsulfonephthalein from  $P_H (= -\log H \text{ concentration}) = 7-8$ , and a similar set of tubes filled with Sørensen's borate buffer mixtures plus thymolsulfonephthalein<sup>1</sup> (Lubs and Clark) from  $P_H = 8-10$ , and sealed in the flame, were used. The phosphate and borate mixtures were calibrated with the hydrogen electrode, but owing to the salt action on the indicator 0.3 must be subtracted from the  $P_H$  of sea water as determined with the tubes in order to obtain the same result as with the hydrogen electrode. Although the  $P_H$  of sea water was determined with the hydrogen electrode out to the second place of decimals

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<sup>1</sup> For the convenience of Dr. Rowntree, Dr. A. G. Mayer, and others I have standardized solutions or samples in relation to the phenolsulfonephthalein and thymolsulfonephthalein tubes for determining hydrogen ion concentration kept in stock for sale by Hynson, Westcott and Co., Baltimore.



(0.5 millivolt = 0.008  $P_H$ ), the estimation of the salt action on the indicator could not be carried this far because the tubes read only to the first place of decimals. Palitzsch defines the  $P_H$  to the second place of decimals, but this must be an estimate since his borate mixtures record only to the first decimal place. The apparently remarkable coincidence that the correction for salt action (at 35 to 36 salinity) should be the same for phenol-sulfonephthalein and thymolsulfonephthalein is probably due to the similar chemical constitution of these indicators. The advantage of these indicators over  $\alpha$ -naphtholphthalein and phenolphthalein is that each shows two strikingly different colors, and errors due to dye concentration cannot be hidden.

These tubes of thymolsulfonephthalein are to be especially recommended for determinations at sea in oceanographic work and all determinations of the  $P_H$  of sea water in which an accuracy of 0.1 is sufficient. They were used by Dr. L. R. Cary to measure the  $CO_2$  excreted by medusæ (see also Haas). For a greater accuracy the tubes would have to be made larger or of extremely uniform bore. In making the small tubes the glass should be carefully selected. The bore should be 10 mm.  $\pm$  0.25 mm. They should be filled with 3 cc. of the buffer mixture plus 0.2 cc. of 0.1 per cent thymol-sulfonephthalein in 70 per cent alcohol, measured with a graduated pipette. A test-tube of 10 mm. bore should have an etched mark at 3.25 cc. for use with sea water, since the addition of the sea water after the indicator helps mix the latter. By retaining the 0.2 cc. pipette used in filling the tubes, the necessity of the calibration of one for making the tests is avoided.

The  $P_H$  of Tortugas sea water (within 8 miles of Loggerhead Key or 82° 52' to 82° 58' W. and 24° 30' to 24° 38' N.) from the surface to a depth of 35 meters, as well as in the moat of Fort Jefferson, was found to vary from 8.1 to 8.22, which is about the average for ocean water (7.95 to 8.25, Palitzsch;  $P_H$  of 8.06 just colors phenolphthalein, at 35 salinity). Since there was no general drift of  $P_H$  during the season, it is probable that the limits of variation during the entire year are not very different. No relation between  $P_H$  and location, time, or tide was found.

Since calcium is the only non-volatile base in sea water that has been shown to vary independently of the salinity, it is possible that variations in the calcium content due to the activity of corals and other organisms might affect the  $P_H$  but this question must be reserved for future study. Since I found only 8.5 parts of ammonia per billion, this factor must be excluded. The effect of denitrification is probably small, since I found only minute traces of nitrates in the water. The amount of nitric acid brought down by the rain in the Barbados was estimated by Harrison and Williams at 2.75 kg. per hectare per annum, but this was accompanied by 1.13 kg. of ammonia, which would mean a mixture of  $NH_4NO_3$  and  $NH_4CO_3$ . After

thunder showers over the sea this fixed nitrogen is probably taken up very quickly by algae and denitrifying bacteria. Although the evaporation is rapid, it would not be sufficient to concentrate the fixed nitrogen before it mixed with the sea to a great depth, since Dole could detect no immediate influence of rain on the surface salinity.

That variations in the amount of  $\text{H}_3\text{PO}_4$  in the sea water could account for variations in the  $P_{\text{H}}$  seems improbable, since I found about 4 mg. in 20 liters that had been acidified with HCl and evaporated to a small quantity. Although some phosphates may have been occluded in the salts crystallizing out, this fraction was probably small, owing to the strongly acid reaction which was constantly maintained during the evaporation.

Since dilution of the sea water must dilute the carbonates and bicarbonates responsible for its reaction, it would theoretically affect the  $P_{\text{H}}$ . Solutions of soda were aerated by drawing moist air through them vigorously for 72 hours in order to make their  $\text{CO}_2$  tension equal that of the air. The  $P_{\text{H}}$  of the 0.1 N solution was 9.8; that of the 0.01 N, 9.26; and of the 0.001 N, 8.3. We thus see a marked change in the  $P_{\text{H}}$  with dilution, but the effect of diluting a bicarbonate solution is much less if it is not aerated; we cannot say that the sea is thoroughly aerated, and, furthermore, the presence of neutral salts in the sea affects the dissociation of the alkali. In order more nearly to imitate the effect of rain falling in the sea, a portion of sea water of  $P_{\text{H}} = 8.1$  was diluted with an equal volume of conductivity water of  $P_{\text{H}} = \pm 6$ , and the  $P_{\text{H}}$  of the mixture was found to be about 8.09 in the hydrogen electrode. Perhaps if the  $\text{CO}_2$  tension of the conductivity water had been exactly that of the air, a greater change would have been noted, but the experiment indicates that a dilution of the most concentrated Tortugas sea water (salinity = 36.09) to the most dilute (salinity = 35.23) would not account for the variation in  $P_{\text{H}}$  (from 8.22 to 8.1).

There remains to be considered the change in  $\text{CO}_2$  content and its effect on the  $P_{\text{H}}$  of the sea water. No direct measurements of the  $\text{CO}_2$  content of the sea water were made, but the change of  $P_{\text{H}}$  with change of  $\text{CO}_2$  tension is shown in Fig. 2. In making mixtures containing less than 1 per cent  $\text{CO}_2$ , a double dilution is necessary. For instance, in making 0.02 per cent  $\text{CO}_2$  a 1 per cent mixture is made in the combined tonometer and hydrogen electrode as previously described (McClendon and Magoon), then the apparatus is shaken (without disconnect-

ing the mercury funnel) so as to mix the  $\text{CO}_2$  thoroughly with the  $\text{H}_2$ . By raising the mercury funnel, all but 2 per cent (2 cc.) of the gas mixture is expelled, then hydrogen is admitted in the usual manner so that the resulting mixture contains 0.02 per cent

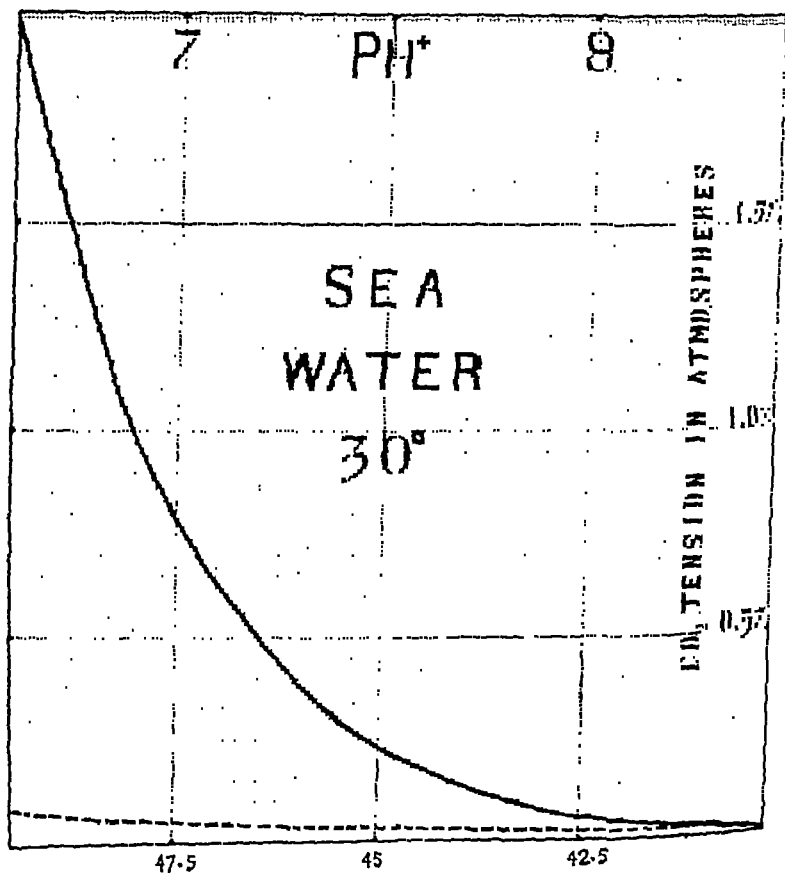


FIG. 2. Table for converting  $P_{\text{H}}$  of sea water (35 salinity) into  $\text{CO}_2$  tension and total  $\text{CO}_2$  content at  $30^\circ$ . The dotted line represents cc. of total  $\text{CO}_2$  per liter.

$\text{CO}_2$ . After shaking 1.5 cc. of sea water with 10 cc. of a gas mixture in the tonometer, the  $\text{CO}_2$  content of the gas mixture may be perceptibly changed, so it was necessary, after determining

the  $P_H$ , to expel the gas mixture and make a new one like it, and repeat this process until a constant  $P_H$  was reached.

The change in  $P_H$  with a change in  $\text{CO}_2$  tension is shown in the continuous line in Fig. 2. The dotted line shows the total  $\text{CO}_2$  content extrapolated from data given by Fox, and is 40 cc. at 0.015 per cent  $\text{CO}_2$  tension, 41.9 at 0.02 per cent, 44.5 at 0.03 per cent, 46.25 at 0.04 per cent, and 47.5 at 0.05 per cent.

Fig. 2 shows that within the normal limits for sea water a very small change in the  $\text{CO}_2$  tension or  $\text{CO}_2$  content causes a comparatively large change in the  $P_H$ , and the changes that have been observed in the  $P_H$  of uncontaminated ocean water could probably be explained by the changes in  $\text{CO}_2$  tension produced by the action of organisms. I found that a jellyfish pulsating in sea water in a closed vessel changed the  $P_H$  from 8.2 to 8 in a very short time, indicating an increase of about 6.5 cc. of  $\text{CO}_2$  per liter. Algæ in the sunlight would change the  $P_H$  to 8.25 in less time. It seems almost certain that the  $\text{CO}_2$  tension is the chief factor in determining the  $P_H$  of the oceans. If this is true, the water from a depth of 2,000 meters in the Atlantic, which Palitzsch found to have a  $P_H$  of 7.95, should have a  $\text{CO}_2$  tension of about 0.06 per cent at  $30^\circ$ , and Tortugas water should have a  $\text{CO}_2$  tension of about 0.03 to 0.043 per cent. In one experiment water drawn directly from the sea into the electrode had a  $P_H$  of 8.15. It was then passed into the tonometer and its  $P_H$  at different  $\text{CO}_2$  tensions determined, with the result that it had a  $P_H$  of 8.15 at 0.04 per cent  $\text{CO}_2$  tension, from which I conclude that this was the  $\text{CO}_2$  tension of the sea where the sample was taken.

It would be interesting to know the  $\text{CO}_2$  tension of the air at Tortugas to see how it compared with the sea. An attempt was made to determine this by bubbling air through the water until equilibrium was reached and then determining the  $P_H$  of the water. The first experiments were valueless, owing to the assumption that equilibrium would be reached within a few hours. It was found necessary to pass a strong stream of moist air through a small quantity of sea water at least 15 hours in order to reach equilibrium, and during this time the temperature changed about  $5^\circ$  if the aeration was all done continuously. In one experiment starting with  $P_H=8.1$ , equilibrium was reached at the end of 15 hours' aeration with the final temperature  $30^\circ$ , and  $P_H=8.16$ . After 24 hours' more aeration (which was stopped at a lower temperature), the  $P_H$  was about the same (8.15).

We may assume, therefore, that the  $\text{CO}_2$  tension of Tortugas air on that day was about 0.033 per cent. Legendre found the air off the coast of France to contain 0.022 to 0.087 per cent  $\text{CO}_2$ , and Lewy found ocean air in the Antilles to contain from 0.038 to 0.053 per cent  $\text{CO}_2$  (average 0.046 per cent). He found ocean air to average 0.053 per cent in the day and 0.035 per cent at night, presumably due to absorption of  $\text{CO}_2$  by the water when cooled at night.

Since the sea contains more than thirty times as much  $\text{CO}_2$  as the air, it must regulate the average concentration of this gas in the air. Krogh supposes that  $\text{CO}_2$  is being increased in the air by the combustion of coal and hence the  $\text{CO}_2$  tension of the air is greater than that of the sea. He calculates the carbon in the air as 600 billion tons, and since the annual consumption of coal is now more than a billion tons, the increase might become measurable if it were not absorbed by the sea. Chamberlin supposes that 440 million tons of carbon are withdrawn annually from the air by the weathering of rocks.

If the  $\text{CO}_2$  content of the Gulf Stream water remains approximately constant as it flows northward, its  $\text{CO}_2$  tension must decrease, owing to the increase in the absorption coefficient for  $\text{CO}_2$  and the decrease in the dissociation of bicarbonates with fall in temperature. But if equilibrium with the air is partially reached, the  $\text{CO}_2$  content and  $\text{H}^+$  concentration should be greater in the North, which seems to be the case. Palitzsch found the  $P_{\text{H}}$  in the Atlantic equal to 8.1 in the far North, and 8.25 nearer the equator. If the sea locally affects the  $\text{CO}_2$  content of the air, we might expect northern air to be poor in  $\text{CO}_2$ , but Krogh found from 0.025 to 0.07 per cent in the air of Greenland, whereas the average for the world is taken at 0.03 per cent. Benedict found, however, from 0.01 to 0.034 per cent between Boston and Genoa, and 0.003 to 0.027 per cent between Montreal and Liverpool, with the same apparatus. It would be easier to determine whether  $\text{CO}_2$  is being absorbed or given out by the sea by determining the  $\text{CO}_2$  tension of the sea and air simultaneously at the same place, as I hope to do next year.

The sea water is so complex a mixture that it would be difficult to apply the law of mass action to it, but owing to the remarkable constancy in the relative amounts of the chief neutral salts in it, we may say that the  $P_{\text{H}}$  depends on the excess of non-volatile base over acid and also on the  $\text{CO}_2$  tension, at a given temperature. This excess of base was found by Dole to be 0.00237 to 0.00257 N, but this determination seemed of sufficient importance to see whether the same result is obtained by other methods of titration as follows: The titrations were made with 0.1 N HCl. The sea water (100 cc.) was placed in a wide-mouth bottle covered by a paraffined cardboard, perforated in three places for the introduction of the burette tip, the hydrogen electrode used as

an indicator, and a rubber tube of 1 mm. bore filled with saturated KCl solution and closed at the end with a wooden plug, for connection with the calomel electrode. After dropping in a small quantity of acid a vigorous stream of hydrogen was passed through the electrode into the sea water, which was allowed to fill the electrode and was blown out with hydrogen three times; it was then allowed to rise only far enough to touch the platinized disk, and the reading was taken. This process was repeated, and the curve of  $P_H$  and cc. of acid was plotted. The angle of the curve was taken to denote the acid necessary to neutralize the excess of base over non-volatile acid. I found the excess base in sea water to be 0.0023 to 0.0025 N. Titrations with 0.05 N  $H_2SO_4$  gave the same results, but the angle seemed not to be as sharp as with HCl. That the excess base had been neutralized at the angle in these titration curves was confirmed by adding the indicated amount of acid to 100 cc. of the same sample of sea water and determining the  $P_H$  after expelling the  $CO_2$  more or less completely. After bubbling air through for 12 hours, the  $P_H$  was about the same as that of a NaCl solution that had been exposed to the air. After boiling it was nearly neutral, and after boiling down to half volume it was also about neutral, and the same was true after bubbling hydrogen through it for a long time. In no case was it alkaline. Artificial sea water made of absolutely neutral salts plus 24 cc. of 0.1 N  $NaHCO_3$  to the liter reached a  $P_H$  of 8.2 after aeration for about 6 hours, whereas if only 23 cc. were used the aeration could be continued for a slightly longer period without danger of making it too alkaline. Since artificial sea water containing only 23 cc. of the soda solution reacted to aeration more nearly like natural sea water, this amount of soda (added as bicarbonate) is recommended in making artificial sea water, which should be aerated sufficiently to bring the  $P_H$  to about 8.15.

Although Herbst maintained the life of sea urchin eggs for some time in artificial sea water, and the experiments of many investigators have shown that the exact proportions of the salts are immaterial in many physiological experiments, Dr. A. G. Mayer informed me that no artificial sea water had been found that would maintain the jellyfish, *Cassiopeia*, in a normal condition. It pulsates intermittently (after the excitement has passed)

when put into artificial sea water and continues to do so until removed or until death ensues. It seemed important, therefore, to attempt to make a more successful sea water in order to determine, if possible, the full physiological significance of this medium. The principal salts are so well known that errors in their concentration would not be looked for in locating the trouble. The minor constituents are probably more variable, and the older analyses not so reliable, but some more accurate analyses have been made by Raben, Matthews, and others. It is possible that organisms may suffer from lack of some of these minor constituents, and it is also possible that organisms may suffer from an excess of them when they occur as impurities in the principal salts or in the distilled water. These constituents are supposed to occur as follows, in parts per million:<sup>2</sup>

NH <sub>3</sub> .....	0.0085-0.15	Cu.....	0.012(?)
Li.....	Trace.	Ag.....	0.01-0.169
Rb.....	11-15(?)	Au.....	0.005-0.035
Cs.....	Trace.	Ra.....	0.000000017(?)
Sr.....	"	F.....	0.3-0.8
Ba.....	"	I.....	0-2.19
Mn.....	"	NO <sub>3</sub> +NO <sub>2</sub> .....	0.18-1.1
Zn.....	0.002	PO <sub>4</sub> .....	0.2-2.2
Fe.....	0.9-3.0	SiO <sub>2</sub> .....	0.2-1.4
Co.....	Trace	Al <sub>2</sub> O <sub>3</sub> .....	0.2(?)
Ni.....	"	B.....	Trace.
Pb.....	0.1(?)	As.....	0.01-0.08

If the purest reagents are used for the principal salts in making sea water, the mixture would be supposed to contain, in parts per million, about: 0.03 Al<sub>2</sub>O<sub>3</sub>; 0.3 SiO<sub>2</sub>; 0.2 Fe; 0.05 I; traces of Ba and NH<sub>3</sub>; traces of As and heavy metals; 0.001 NO<sub>3</sub>; and 0.0003 PO<sub>4</sub>. Warburg found enough Cu in reagents that had been crystallized in copper dishes to increase the oxidation greatly in sea urchin eggs. Warburg found further that the addition to sea water of 0.001 Ag or 0.002 Au or 0.0006 Cu (parts per million) increased oxidation in unfertilized sea urchin eggs about 600 per cent.

<sup>2</sup> For references to the literature on this subject see the works of Clarke, Krümmel, Raben, Matthews, Roth, Murray and Hjort, Forchhammer, and Dittmar, quoted in the list of references at the end of this paper.

In order to test whether there might be enough heavy metals in "reagent," "analyzed," and "*für Analyse*" salts to affect organisms, sea water was made from them and compared with that made from further portions of the same salts after solution in water redistilled in quartz (since Locke found Cu in distilled water in a toxic concentration) and recrystallization in "pyrex" glass. The NaCl was precipitated by admitting HCl gas into its saturated solution. Instead of  $\text{NaHCO}_3$ , recrystallized  $\text{Na}_2\text{CO}_3$  plus an excess of  $\text{CO}_2$  was used. The results were equally favorable with the two sea waters. The addition of as much as five parts per million of  $\text{PO}_4$  was detrimental to animals.

Since  $\text{H}_2\text{O}$  is the chief constituent of sea water, particular attention was paid to it. It was found that conductivity water made in Hulett's laboratory and sealed in pyrex flasks by fusing the glass accomplished no better results than Merck's distilled water. Pyrex glass contains arsenic, but very little should have dissolved in the water since the glass is extremely insoluble. It is said to contain no lead. The only positive result from experiments with different qualities of distilled water was that aeration improves it. The aeration necessary to bring the artificial sea water to the required  $P_R$  is not sufficient from other standpoints. It was not determined whether the beneficial effect of aeration was the addition of some element ( $\text{O}_2$ , for instance), or the elimination of gaseous impurities. Merck's distilled water was free from  $\text{NH}_3$  but had a taste that was not lessened by redistillation in quartz. The conductivity water had this taste in a much smaller intensity, and it required 72 hours of vigorous aeration to reduce the taste (of a liter) of Merck's water to equal that of conductivity water. Since the conductivity water had purposely been kept from the air as much as possible, its lack of taste may have been due to the oxidation of all organic impurities. If this was so, the taste of Merck's water must have been due to some volatile substance other than  $\text{NH}_3$  or  $\text{CO}_2$ .

In making the artificial sea water, the salts were made up in normal solutions (0.5 M of bivalent salts), and the following number of cc. were used to make a liter, according to an analysis of Tortugas sea water of salinity 35.49 (Clarke) or 35.41 (Dole). The proportions for isotonic solutions are also given. The



isotonic solution of  $\text{NaHCO}_3$  is approximately isotonic after aeration, but it should be noted that these solutions cannot be exactly isotonic since mixing them affects the dissociations.

	Normal solutions.		Isotonic solutions.	
		cc.		cc.
$\text{CaCl}_2$ .....	0.5 M	22.0	0.38 M	29.0
$\text{MgCl}_2$ .....	0.5 M	50.21	0.37 M	67.9
$\text{MgSO}_4$ .....	0.5 M	57.09	0.975 M	29.5
$\text{KCl}$ .....	M	10.23	0.577 M	17.7
$\text{NaCl}$ .....	M	483.65	0.568 M	852.0
$\text{NaBr}$ .....	M	0.8	0.565 M	1.4
$\text{NaHCO}_3$ .....	M	2.32	0.930 M	2.5
$\text{H}_2\text{O}$ .....		373.63		
		1,000.00		1,000.0

The mixture should be aerated until it has a  $P_H$  of about 8.15.

This artificial sea water was tested with all the delicate marine organisms available. The lagoon jellyfish, *Cassiopeia xamachana*, lived in it indefinitely and its pulsations were normal. Some died in 6 weeks if the water was allowed to evaporate, but the prevention of evaporation or the occasional addition of distilled water to restore the volume was all that was necessary to keep them alive (without food). Eggs of the Atlantic Palolo worm, *Eunice fucata*, developed in it and produced two pairs of setæ. Some were alive at an age of 13 days, although they had never been fed.

Many animals apparently live at a great depth in the sea where the water is cold, and when brought to the surface in warm regions die sooner or later, and very rapidly when brought into laboratories. Since these animals are considered to be the most delicate, I placed them in the artificial sea water. *Sagitta* lived 7 days, various pelagic medusæ, ctenophores, and siphonophores lived about 12 hours when crowded in small vessels, and *Salpa* only a few hours under the same conditions. If the  $P_H$  was changed beyond the limits, 6 to 8.26, death of all of the animals occurred sooner.

The  $P_H$  of sea water is rapidly changed by the presence of animals or eggs, even in uncovered dishes. For this reason it seemed

of interest to determine the  $P_H$  of the fluids inside invertebrates, which do not differ markedly from sea water in the salts they contain, and are often very poor in proteins. The  $P_H$  of the body fluid of the sea urchin, *Toxopneustes* (*Lytechinus*) *variegatus*, was found to vary from about 7.7 to 7.8. The blood of the conch, *Strombus gigas*, was about 7.5.

Some salts may be considered nutritive, especially to plants. The growth of marine Diatoms, but not algæ, was favored when I added 2 cc. of M  $\text{Ca}(\text{NO}_3)_2$  per liter. But most of the salts, or more correctly, ions in sea water, are to be considered protective rather than nutritive, especially to animals. Since the work of Ringer, O. Loew, Loeb, Mayer, and others, the idea that one ion protects the organism from the toxic action of another ion has constantly gained ground. I have shown that certain ions increase the permeability of sea urchin and fish eggs (McClendon, 1910, 1914; McClendon and Mitchell). Since these ions are present in sea water they must be antagonized by other ions in sea water. This work was greatly extended by Osterhout who showed that certain ions increase the permeability of plant cells and others inhibit their action. If the action of all ions is of this nature there must be only two classes of ions, those that increase permeability and those that inhibit this change. The work of Ringer, Mines, and others shows the antagonistic action of ions on the heart. In order to test this hypothesis of the two classes of ions the action of ions of sea water on the pulsations of *Cassiopeia* and the heart of the conch was studied. I had found the freezing point of a sample of Tortugas sea water in 1910 to be  $-2.03^\circ$  when corrected for undercooling, which is the freezing point calculated from a salinity of 37, whereas that of salinity 35.41 is  $-1.937^\circ$ .<sup>3</sup> Isotonic solutions were made as follows:

$\Delta = 1.937^\circ$ : 0.565 N NaCl, 0.577 N KCl, 0.378 M  $\text{CaCl}_2$ , 0.364 M  $\text{MgCl}_2$

$\Delta = 2.03^\circ$ : 0.59 N NaCl, 0.6 N KCl, 0.395 M  $\text{CaCl}_2$ , 0.38 M  $\text{MgCl}_2$

Whole *Cassiopeias* when paralyzed by pure  $\text{MgCl}_2$  solution begin to beat after the addition of a little KCl or more NaCl, but not by  $\text{CaCl}_2$ . When paralyzed by a pure  $\text{CaCl}_2$  solution they may begin to beat if sufficient NaCl or KCl is added before this toxic

<sup>3</sup> Krümmel, page 241.

solution has had time to injure the animal severely. This action of Na is inhibited by an increase in the concentration of H. The rate of pulsation is above normal in pure NaCl solution, but is reduced (finally to zero) by increase in the H<sup>+</sup> concentration. If the  $P_H$  of sea water is changed beyond the limits, 7.5 to 8.25, the pulsation rate is finally reduced, but a long period may intervene before the pulsations respond unless the change in  $P_H$  is great.

The pulsation rate is increased by the addition of KCl to sea water, and its action is inhibited by increase in H. If the *Cassiopeia* is paralyzed by a marked increase in the H<sup>+</sup> concentration of sea water it may beat after the addition of KCl. When paralyzed by MgCl<sub>2</sub> or CaCl<sub>2</sub> it may be caused to beat by increasing the OH<sup>-</sup> concentration.

The heart of the conch will beat if perfused with sea water. In these experiments the auricular wall was used to make connection with a glass reservoir, and a hydrostatic pressure of about 2 cm. of water was substituted for the auricular action; hence only the ventricular action was studied. It stops in systole if the  $P_H$  of the sea water is changed to 9.7, and in diastole if the  $P_H$  is 5.6. Sea water of  $P_H = 8.26$  deposits CaCO<sub>3</sub> on glass, and a precipitate occurs throughout the solution if the alkalinity is increased much further, but it may remain in a supersaturated condition, as regards CaCO<sub>3</sub> for some time. It is improbable that a precipitation of CaCO<sub>3</sub> occurs in the tissue before it appears generally, since the tissue is constantly reducing the alkalinity by the production of CO<sub>2</sub>. Within the limit of  $P_H = 9.5$ , an increase of OH<sup>-</sup> merely increases the rate of pulsation. The effect of the ions may be summarized: Increase in concentration of OH<sup>-</sup>, Na<sup>+</sup>, or K<sup>+</sup> increases the rate and finally stops the heart in systole; whereas H<sup>+</sup>, Mg<sup>++</sup>, and Ca<sup>++</sup> decrease the rate and may stop the ventricle in diastole. This action of Ca is only seen when it is applied suddenly in great excess, since the discovery of Ringer that Ca<sup>++</sup> favors systolic contractions holds true for the conch ventricle. When placed in equal parts of sea water and isotonic CaCl<sub>2</sub> solution, the ventricle stops in diastole but soon shrinks up and cannot be revived. In the following tabulation, the numbers express the volumes of neutralized sea water (n. s. w.) to which one volume of isotonic chloride solution is

added, and the effect produced, followed by the effect of the addition of another solution.

4 n. s. w. + Na increases rate, + HCl to make 0.001 N, stops in diastole.  
 11 " + K " " , + HCl " " 0.001 N, " " "  
 4 " + Mg stops in diastole, + OH<sup>-</sup>, starts again.  
 1 " + Ca " " " then passes into rigor.

We may sum up these experiments on *Cassiopeia* and the conch ventricle in the light of electrical conductivity experiments on animals and plants by assuming that Na<sup>+</sup>, K<sup>+</sup>, and OH<sup>-</sup> increase the permeability of the plasma membrane and that Ca<sup>+</sup>, Mg<sup>+</sup>, and H<sup>+</sup> inhibit their action, thus causing decrease in permeability when the antagonistic ions are present. It has been shown that muscular contraction is accompanied by increase in permeability (McClendon, 1912) which may explain the fact that Na<sup>+</sup>, K<sup>+</sup>, and OH<sup>-</sup> favor systole, and Mg<sup>+</sup> and H<sup>+</sup> favor diastole, but the experiment must be performed in a certain way in order to show that Ca<sup>+</sup> may produce diastole. The anomaly that a small amount of Ca<sup>+</sup> favors the systolic contractions produced by a large excess of Na<sup>+</sup> may be explained by the assumption that the optimum ratio of each pair of antagonistic ions is different for each part of an organism and that more than one of these hypothetical parts are concerned in any one of the experiments. For instance, suppose the optimum ratios are as follows, Ca: Na = 1:100 for muscle, 1:50 for nerve fiber, 1:25 for motor end plate, 1:10 for nerve cell body, it is clear that no ratio of Ca to Na would be especially favorable for an action in which all of these structures take part. In sea water we have a summation of antagonisms in which Na<sup>+</sup>, K<sup>+</sup>, and OH<sup>-</sup> are combined against Ca<sup>+</sup>, Mg<sup>+</sup>, and H<sup>+</sup>, and the proof that it is favorable for all of these structures is that it works. It should be added that some of the minor constituents of sea water take part in these antagonisms, and that their effect is large in comparison to their concentration.

Since the statement was made by Mines that the heart of a species of *Pecten* would not beat in sea water unless it is neutralized or slightly acidified, it seems worth noting that this is not general even for molluscs of this particular group. The heart of *Pectenella* will beat in normal, neutral, acid, or hyperalkaline sea water, and even in hyperalkaline NaCl solution.

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## DIETARY DEFICIENCIES OF THE MAIZE KERNEL.\*

By E. V. McCOLLUM, N. SIMMONDS, AND W. PITZ.

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(Received for publication, October 23, 1916.)

It is a well known fact that young pigs cannot grow when confined to the maize kernel as the sole source of nutriment,<sup>1</sup> and the reasons for the failure have now been made approximately clear. We have seen pigs grow well during several months when limited to a mixture of corn kernel (maize) 70 per cent and gluten feed 30 per cent (maize), supplemented with a suitable inorganic salt mixture when distilled water only was supplied. Without the salt additions early cessation of growth, loss of appetite, stiffness of joints, and roughness of coat revealed a serious condition of malnutrition. When given natural water and allowed access to the soil nearly normal growth was observed, during several months without salt additions.

In the present paper we present the results of a systematic series of experiments with ground maize in which single or multiple additions of purified food substances were made. These included protein, inorganic salts, and butter fat to supply the unidentified dietary factor, the fat-soluble A. Numerous experiments have shown that the second as yet unidentified dietary factor, the water-soluble B, is furnished in great abundance by even 70 per cent of maize in the diet (Chart 5).

The method of procedure was similar to that which we have described in connection with our studies of the dietary deficiencies of wheat, rice, and wheat embryo<sup>2</sup> and the results make it clear that the dietary properties of the maize kernel are very closely

\* Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>1</sup> Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

<sup>2</sup> McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615.

similar to those of the wheat kernel. The conclusions may be briefly summarized as follows:

1. The proteins of the maize kernel contain all the amino-acids essential for growth, but the proportions of certain of them are such that they are not utilizable to a high degree as the sole source of protein. When other factors affecting nutrition were properly adjusted, we have seen growth at about two-thirds the normal rate over a period of 6 or 7 months, on a diet in which all the protein was derived from 91 per cent of ground maize in the ration (9 per cent of  $N \times 6.25$ ) (Chart 4).

2. The maize kernel contains both the unidentified dietary factors, the fat-soluble A and water-soluble B. The former is present in amount too small for the maintenance of growth at the maximum rate in rats, and regardless of how satisfactorily the maize kernel is supplemented in other respects, failure of perfect nutrition will supervene within a few months unless some food-stuff containing the fat-soluble A (butter fat, certain other fats, leaves of plants, etc.) is supplied (compare Charts 5, 6, and 7). Hot alcohol extracts the fat-soluble A from the maize kernel. We have supplemented the low content of the dietary A of maize with an alcoholic extract of maize and have induced development more closely approximating the normal than without this addition, followed by reproduction and rearing of the young (Chart 9).

3. Like other grains which we have studied, maize contains an abundance of the unidentified dietary factor, the water-soluble B. This is made evident by the fact that the maize kernel can be supplemented so as to produce normal nutrition by the addition of a suitable purified protein, inorganic salts, and butter fat, none of which carry this dietary factor (Charts 6 and 8).

4. The inorganic content of the corn kernel is not of a character suitable for the promotion of growth. We have found it necessary in all cases to make salt additions to rations deriving their inorganic contents principally from this source, regardless of the nature of the other purified food ingredients added, before growth could take place (Chart 8).

5. The addition of purified protein and salts (Chart 6) or of butter fat and salts (Chart 4) to the maize kernel fails to induce physiological well-being throughout the life of the animal. The

addition of protein and butter fat without salts (Chart 8, Period 1) forms a poorer food mixture than the pairs of additions first named (compare Charts 4 and 6 with Chart 8, Period 1). While we have seen pigs grow during several months when the diet was restricted to the maize kernel fortified with additional maize protein (gluten feed) and inorganic salt additions, perfectly normal reproduction has never been observed on such rations. Young were born but the mother failed to rear them. This ration was directly comparable with that of Lot 569 (Chart 6).

The experiments here reported with rats confirm our observations with swine on certain rations derived solely or principally from the maize kernel and extend the experimental data relative to the quantitative values of the maize kernel as respects the several dietary factors. We shall report later the results of feeding high planes of intake of maize proteins.

We have attempted several times to nourish young rats with a diet restricted to the germ of the maize kernel, and to mixtures of the maize germ and whole ground maize in several proportions. These attempts have been uniformly unsuccessful (Chart 10). Two samples of germ from different dealers were employed. We are not certain just what treatment these had received, but both appeared to be wholesome products. It is evident from these trials that it is not easy, if at all possible, to make up a satisfactory ration wholly derived from the corn kernel and its parts. The nature of the dietary deficiencies of the corn germ constitutes a problem in itself.



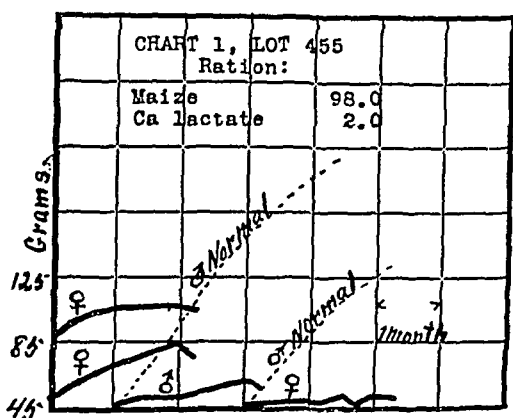


CHART 1. Lot 455 shows the failure of rats to make any appreciable growth when restricted to a diet of ground maize 98 per cent and calcium lactate 2 per cent.

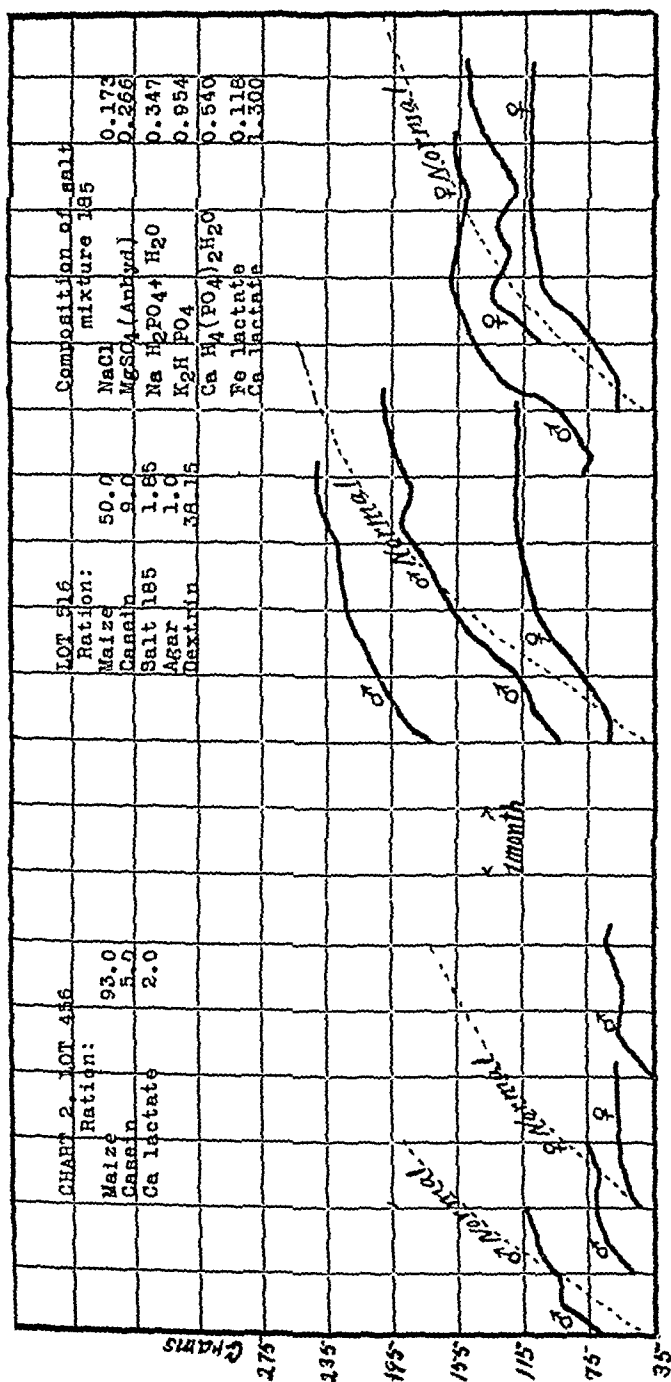


CHART 2. Lot 456 shows further that the character of the proteins is not the sole cause of the failure of rats to grow when restricted to the maize kernel as the only source of nutriment. The addition of 5 per cent of casein exerts little, if any, beneficial effect on growth.

Lot 516 received a still greater addition of casein (9 per cent) and 1.85 gm. of a salt mixture which is highly satisfactory when employed in a ration of purified foodstuffs. There was a marked improvement in growth during the first 60 days, but thereafter the animals remained stunted.

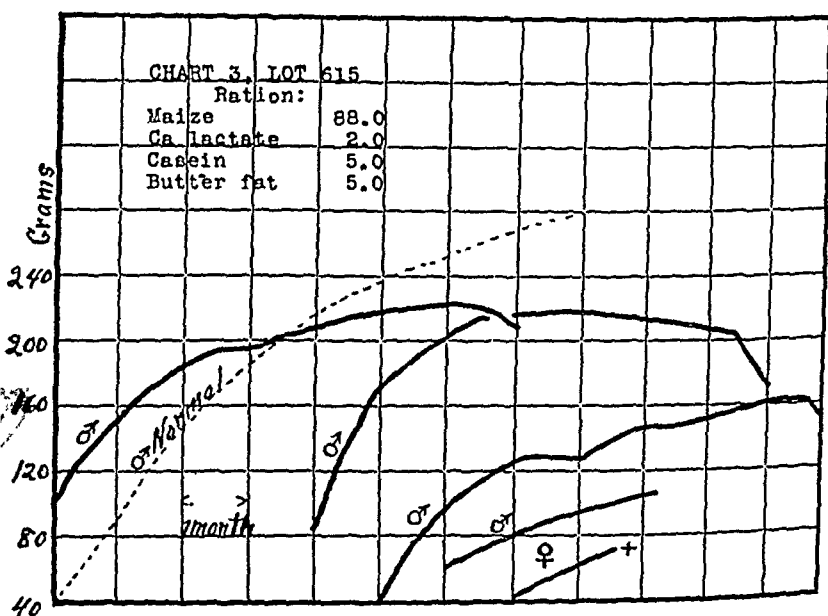


CHART 3. Lot 615 illustrates the fairly satisfactory growth during the first 2 months on a ration closely similar to that of Lot 456, Chart 2, except that 5 per cent of butter fat replaced 5 per cent of maize. All the components of a complete food are present in this ration. Failure to complete growth to the normal adult size shows a lack of proper adjustment among the several dietary factors. Results such as these suggest the explanation of the etiology of the "deficiency diseases." We have elsewhere discussed this question in some detail.<sup>3</sup>

<sup>3</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333.

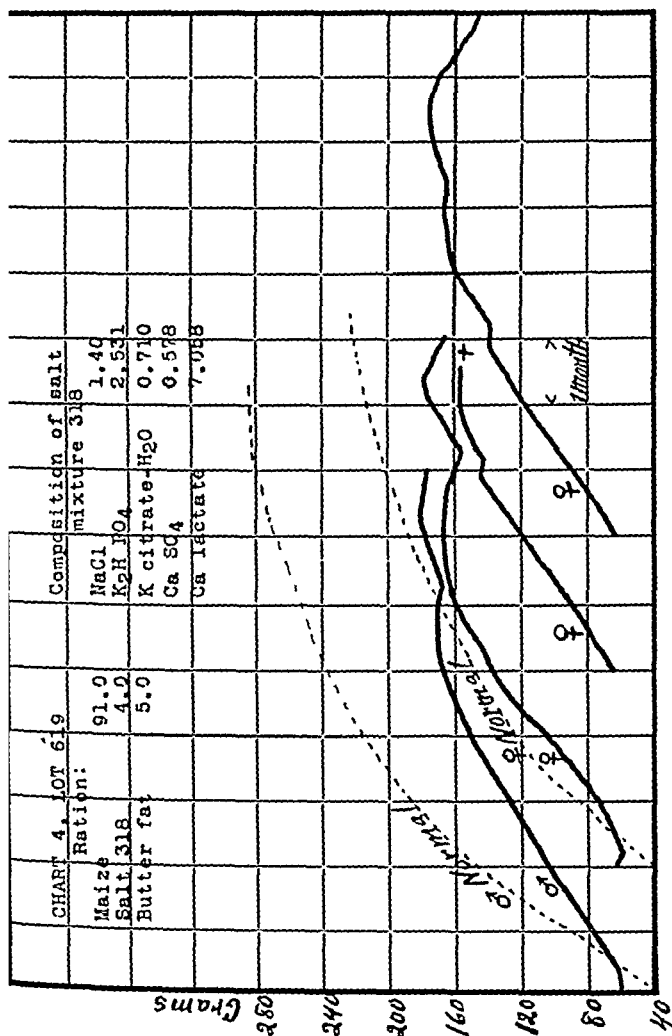


CHART 4. Lot 619 shows that the protein content of the maize kernel is qualitatively adequate for growth, in that it contains all the essential amino-acids. The ration of these rats furnished 9 per cent of protein. This gives evidence of the relatively low efficiency of the maize proteins for growth. The growth secured with this mixture demonstrates the presence of the water-soluble B in the maize kernel.

That the low amount and poor quality of the protein in this food mixture is the cause of unsatisfactory growth is further shown by Lot 568 (Chart 5) whose ration was similar except that a pure protein replaced a part of the maize. This change renders this ration adequate for continued growth.

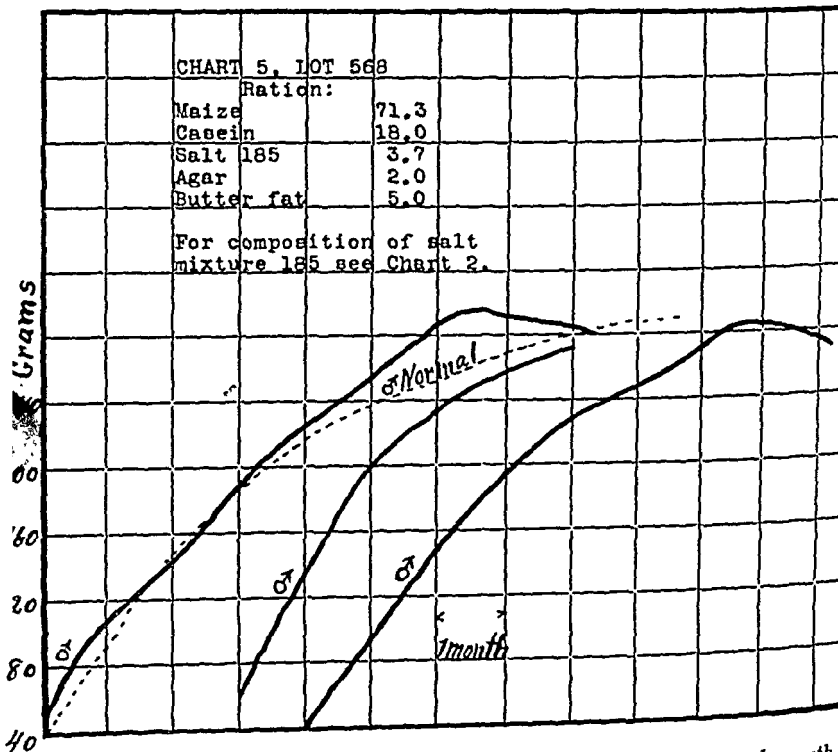


CHART 5. Lot 568 furnishes further evidence that the restricted growth of the rats in Lot 619 (Chart 4) was due to the poor quality of the proteins of the corn kernel. Lot 568 differed essentially from Lot 619 in having a portion of the maize replaced by a pure protein casein. When thus supplemented growth was normal to the usual adult size. That the maize kernel is lacking in the necessary amount of the fat-soluble A is shown by a comparison of the records of the above chart with Chart 6, Lot 569, whose ration differed essentially from that of Lot 568 only in that it contained no butter fat. Without the latter addition, the addition of protein and salts to the maize kernel served only to induce growth at about half the normal rate.

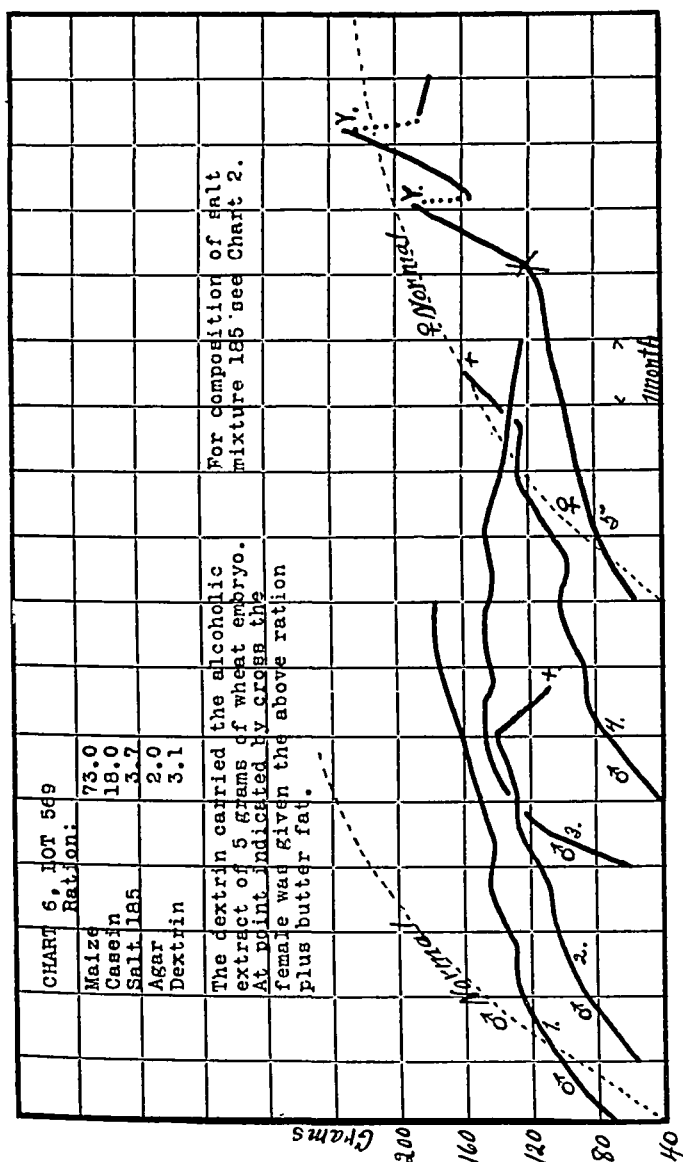


CHART 6. Lot 569 illustrates the inadequacy of the content of the fat-soluble A in the maize kernel. This ration is closely similar to that of Lot 568 (Chart 5) except that it contained no butter fat. With butter fat growth was complete on this food mixture (Chart 5), while without it only very slow growth and stunting resulted (Chart 6). Rat 5 after 5 months on this diet was given 5 per cent of butter fat (ration of Lot 568, Chart 5) with the result that growth was promptly accelerated, and later she produced two litters of young. The first litter was permitted to die, but the second litter of ten young was successfully weaned. At birth the young weighed 48 gm., and at 14 days 145 gm.

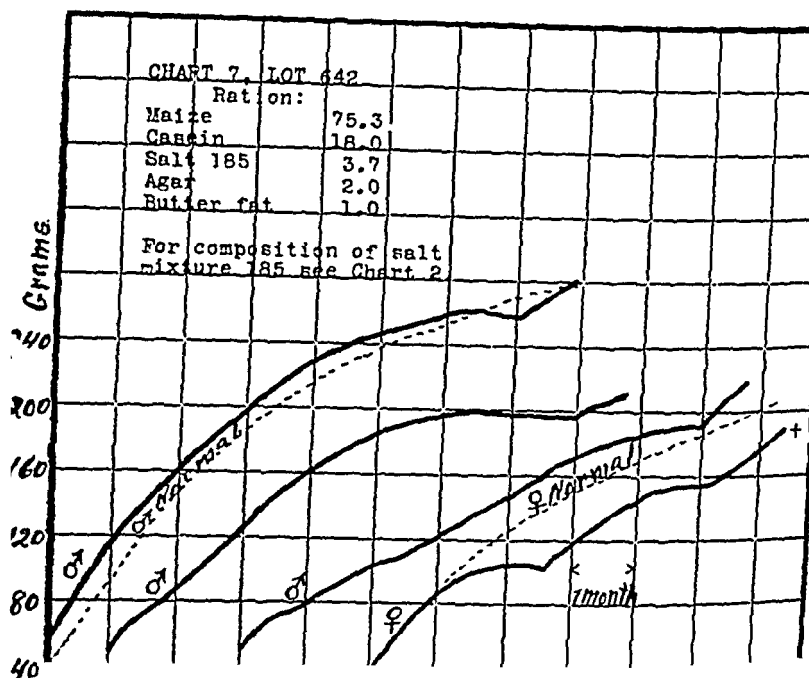


CHART 7. Lot 642 shows, when considered in the light of our experience with rations made up of purified foodstuffs, that the maize kernel contains some of the fat-soluble A, but not enough to support growth. Unpublished data in our records indicate that 1 per cent of butter fat does not supply enough of the indispensable dietary factor, the fat-soluble A, to support growth. This amount when supplemented by the content of the dietary A in 75.3 per cent of ground maize induces nearly normal growth during 4 months. Compare Charts 6 and 7.

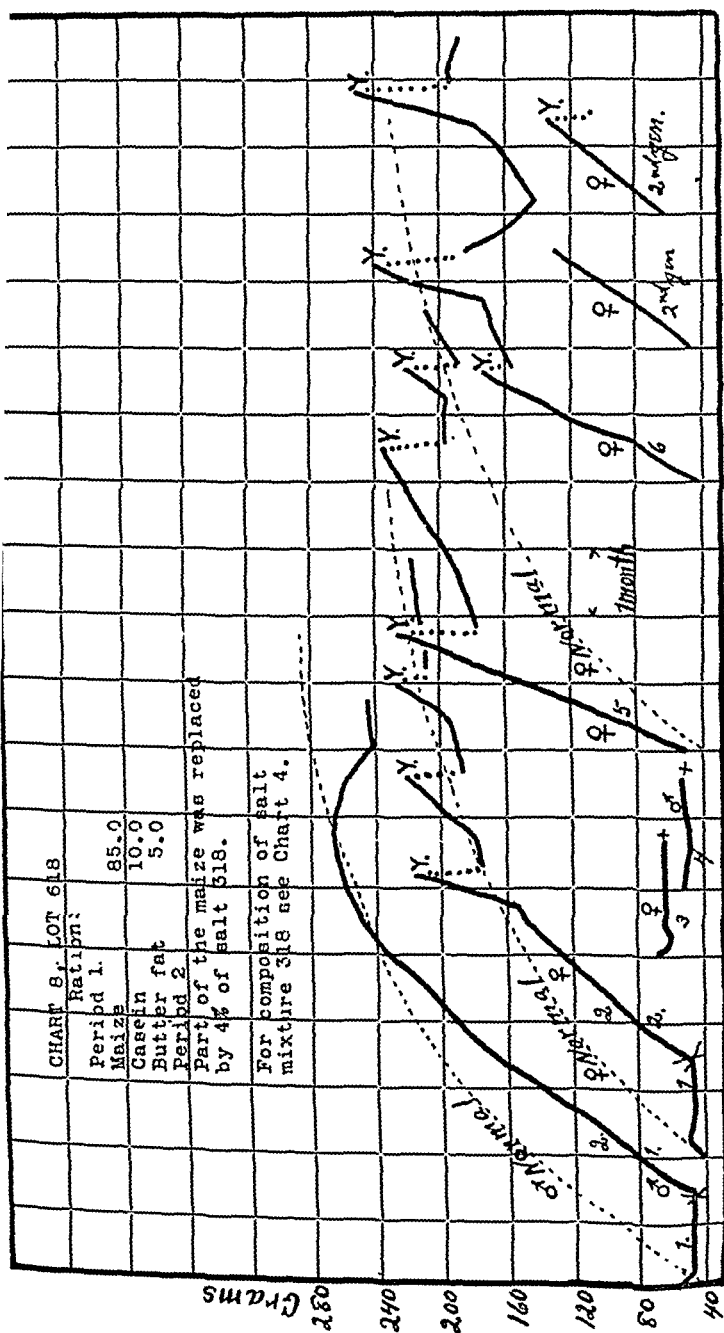


CHART 8. Lot 618 further illustrates the necessity of modifying the inorganic content of the ration by suitable additions when the ration derives its mineral elements principally from the maize kernel. During the first 6 weeks there was no growth on a diet of maize, casein, and butter fat. Growth began at the normal rate, however, when 4 per cent of a suitable salt mixture was introduced into the food (Rats 1 and 2). Rats 5 and 6 received and salt additions from the beginning. Of the nine litters of young born (fifty-eight young in all), three litters (seventeen young) were successfully weaned. They were not normal in appearance; they appeared too short for rats of their weight.



CHART 9. LOT 643

Casein	18.0
Salt	3.7
Maize	6.3

Percentage of alcohol extract of 100 gm. of ground maize in the food mixture, 76.3 per cent.

For comparison of salt mixture, see Chart 2.

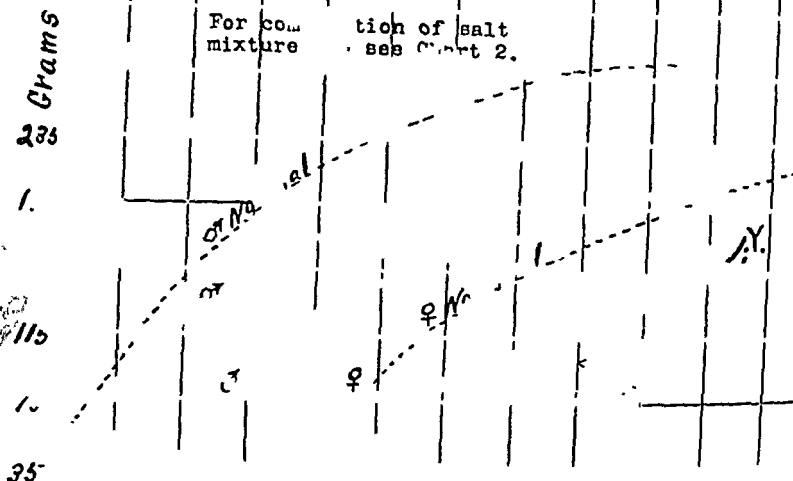


CHART 9. Lot 643 derived all its supply of the fat-soluble A from the maize kernel. Each 100 gm. of ration carried a hot alcoholic extract of 100 gm. of ground maize. This contained enough of the dietary A to supplement that contained in 76.3 per cent of maize in the food mixture, so that the animals were able to grow nearly to the full adult size. After nearly 7 months on this diet the female in this group produced and successfully weaned a litter of four young. The four young at the age of 59 days weighed 213 gm. The records of the rats in Chart 9 furnish conclusive proof of the presence of this dietary factor in the maize kernel.

A point deserving special emphasis which all our experimental data support is the high content of the dietary factor A in the leaf of the plant as contrasted with the seed. 30 to 40 per cent of alfalfa leaves in a grain mixture supplies an adequate amount of this unidentified substance. Considerably more than the content of 100 per cent of maize kernel or wheat kernel is required to induce normal nutrition. While ether does not extract the dietary A with the fats from plant tissues the results of the experiments recorded in Chart 9 show that it is removed from the maize kernel by hot alcohol.

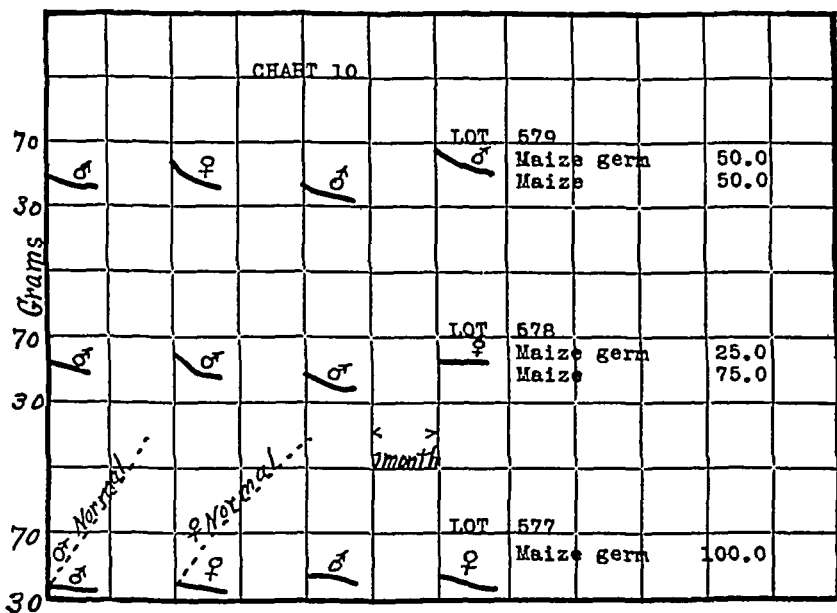


CHART 10. Lots 577, 578, and 579 illustrate the failure of young rats to make any growth on combinations of maize kernel and its germ in various proportions. It is evident that it is not easy, if at all possible, to compound a ration from the maize kernel and its parts which will properly nourish an animal. This grain must be judiciously combined with other foodstuffs to obtain best results.



## A PERFUSION PUMP.

By F. B. KINGSBURY.

(From the Biochemical Laboratories, Department of Physiology, Medical School of the University of Minnesota, Minneapolis.)

PLATES 2 AND 3.

(Received for publication, October 13, 1916.)

Several pumps for perfusing surviving organs have been described in the literature, but it seemed to the writer that a new pump of simpler construction could be devised which would be a distinct improvement on those already existing. Two requirements seem to be of fundamental importance: first, that the material of which the pump cylinders, pistons, and valves are made, the material which comes in contact with the blood or other perfusing liquid, should be of glass or hard rubber, and not of metal, since the use of the latter might introduce complications, either because of the possible toxicity of the metal to living material, or because of the chemical action of the metal upon the perfusing liquid; secondly, it seems safe to assume that pulse pressure is of fundamental importance in maintaining the normal functions of any organ or tissue. Gesell,<sup>1</sup> working with dog kidneys, has recently shown that if the pulse pressure is diminished the functional activity of this organ is distinctly lowered. It seems important, therefore, that a pump to be effective should be capable of imitating the normal pulse pressure curve.

Friedmann<sup>2</sup> has devised two rather elaborate perfusion pumps. The material of which the cylinders, pistons, and valves were made appears to the writer to be metal, from the appearance of the photographic reproduction and because of the difficulty of constructing valves such as Friedmann used out of any other material. One of these pumps is so designed, as appears from the drawings, that it would probably deliver a pulse pressure similar to that of an animal. The other pump is not so designed.

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<sup>1</sup> Gesell, R. A., *Am. J. Physiol.*, 1913, xxxii, 70.

<sup>2</sup> Friedmann, E., *Biochem. Z.*, 1910, xxvii, 87.

The pump described by Richards and Drinker<sup>3</sup> is a distinct advance in that the cylinders, pistons, and valves are made of glass instead of metal. This pump will produce a pressure curve of the right shape. The chief objection to glass is that it is such a difficult material to work with, as pointed out by these investigators. The valves of this pump are rubber tubes which are mechanically compressed in closing, and properly constructed cams insure the correct timing of the opening and closing. I have found that two Bunsen valves, as shown in Fig. 1, serve this purpose adequately since I have been able to produce pulse pressure curves which are similar in form to the normal arterial pulse curve. These curves are shown in Fig. 2.

The two pumps described several years ago by Brodie<sup>4</sup> do not meet the requirements as far as the form of pulse pressure curve is concerned. Hooker<sup>5</sup> has called attention to the fact that a pump in which the crank axle revolves about a fixed point, as is the case with Brodie's pumps, produces a curve having nearly the same velocity of rise and fall, entirely unlike the normal arterial pulse which reaches its maximum quickly to fall off to its minimum slowly. Hooker has made use of a suitable cam and a sliding pump carriage to attain this end. I assume, from lack of knowledge to the contrary, that the cylinders, pistons, and valves of this pump were made of metal.

Since glass is a difficult material out of which to make cylinders and pistons, and because the use of metal for this purpose is inadvisable for the reasons already stated, I have used hard rubber for these parts. Glass valves were first tried, but they proved unsatisfactory, as was also found to be the case by Richards and Drinker, and Bunsen valves were substituted. In this type of valve there is a very slight regurgitation, but this is an advantage over the mechanically operated valves used by other investigators, in the opinion of the writer, since a closer approximation to the normal heart action is attained.

### *Description of the Pump.*

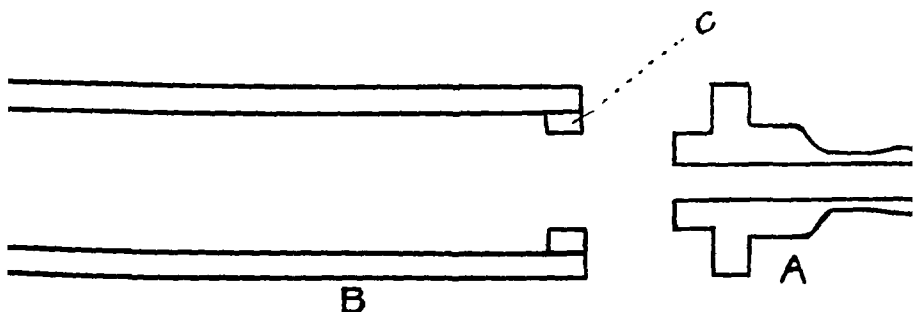
The pump consists of a hard rubber cylinder snugly fitting an enclosing cylinder of brass, threaded on each end to receive brass caps. By screwing these up, the hard rubber nozzle of the pump

<sup>3</sup> Richards, A. N., and Drinker, C. K., *J. Pharm. and Exp. Ther.*, 1915, vii, 467.

<sup>4</sup> Brodie, T. G., *J. Physiol.*, 1903, xxix, 266. Brodie, T. G., and Dixon, W. E., *ibid.*, 1903-04, xxx, 476.

<sup>5</sup> Hooker, D. R., *Am. J. Physiol.*, 1910-11, xxvii, 24.

is held in water-tight contact with the cylinder. Text-fig. 1 illustrates the construction and method of attachment of these parts. The piston, turned to fit the cylinder, is made of hard rubber and has three grooves in it to receive packing thread. The brass piston rod is connected with a cross head of brass traveling on two steel rods. This is attached to a connecting rod of brass which is in turn clamped by a thumb screw to an oscillating steel beam with a slit in it so that the length of pump stroke may be regulated by clamping the head of the connecting rod at the desired place on the beam. The latter is actuated by a rotating cam on the filling stroke of the pump, and by a steel spring on the emptying stroke. The cam is of the proper shape to produce the characteristic pulse pressure curve, and rotates clockwise.



TEXT-FIG. 1.  $\frac{1}{4}$  inch cylinder and nozzle. Actual size. A, nozzle. B, cylinder. C, ring to adapt nozzle to cylinder (hard rubber).

Two sets of pistons and cylinders are provided, one of  $\frac{1}{2}$  inch bore and the other of  $\frac{3}{4}$  inch bore. By means of the former the output of the pump per stroke may be varied from 0.45 cc. to 2.50 cc., and by the latter from 1.00 cc. to 5.70 cc. The steel beam is marked at certain intervals and the corresponding output volumes are known.

The tension of the spring may be regulated by loosening the lock nut on the beam shaft and moving the spring arm forward or backward. As shown in Fig. 3, the screw to which the spring is attached is only temporary, the original design calling for a suitable means of adjusting the spring tension at this point also. The spring must be powerful enough to hold the beam against the revolving cam at all periods of the stroke, re-

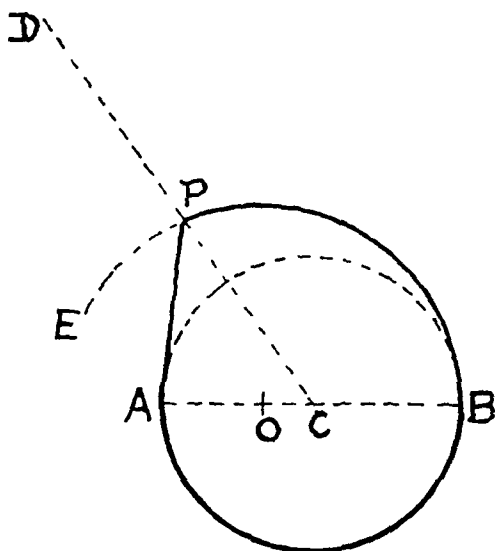
gardless of the length of the stroke and the size of cylinder and piston used. In this case the pulse wave is dependent entirely upon the shape of the cam and is independent of the spring.

As shown in Figs. 3 and 4, the cam shaft is equipped with two pulley wheels of different diameters. Either of these may be belted to any one of four grooves in the driving wheel. The latter is attached to a shaft, driven by a worm gear directly connected with a  $\frac{1}{6}$  horse power direct current motor. The motor is started by means of an eight point rheostat, thus giving eight different motor speeds. The worm gear used by the writer gives a speed reduction of 1 to 80, and the possible variations obtainable are from 14 to 96 strokes per minute. Higher speeds may easily be obtained by using a worm gear of smaller speed reduction, and this is recommended, particularly if the organs of small animals, as cats and rabbits, are to be perfused, since the pulse frequency of these animals may be twice or more than twice the maximum frequency obtainable unless a very large driving pulley is used. The worm gear, driving pulleys, motor, and rheostat are not shown in the figures. As previously noted, the pulse pressure curve produced by the pump is dependent only upon the shape of the cam provided the actuating spring is sufficiently heavy. When the pump is adjusted for a large output per stroke, the spring tension must be much greater than when the output is small. If the spring tension is not sufficient to hold the beam against the rotating cam at all positions, a pulse pressure curve is produced which is more or less dependent upon the spring tension itself since the latter does not expel the liquid from the pump with sufficient velocity to hold the beam against the cam as the point of the latter falls away from it. The writer uses several springs of different degrees of stiffness to fulfil these requirements. The amount of power necessary to run the pump is, *a priori*, greatly increased by increasing the volume of output, and this must be taken into consideration by providing a belt sufficiently heavy to carry the increased load put upon it.

Text-fig. 2 shows the construction of the driving cam. A circle of  $\frac{3}{4}$  inch radius is described with C as a center and the diameter ACB drawn. With O as a center (OB = 1 inch) an arc BE is described. From C a line drawn at 60 degrees to ACB, intersecting arc BE at P. From P a line is drawn tangent to the circle of which ACB is the diameter, completing the cam.

Text-fig. 1 shows the  $\frac{3}{4}$  inch cylinder and its nozzle. The same nozzle fits the  $\frac{1}{2}$  inch bore cylinder also. In the assembled pump, shown in Figs. 3 and 4, the nozzle and cylinder are held in water-tight contact by the two brass caps which screw on to the ends of the brass supporting cylinder. It is impossible for the perfusing liquid to come in contact with the brass caps or brass cylinder, or in fact with any metallic part.

Fig. 1 shows the two cylinders, two pistons, nozzle, and the two Bunsen valves. The latter, simply rubber tubes having short



TEXT-FIG. 2. The cam. Actual size.

longitudinal slits in them, closed at one end with short pieces of glass rod, are attached to the ends of a Y-tube. This is connected by a rubber tube which is short to lessen the amount of dead space in the pump and connections.

Fig. 2 shows kymograph records at different drum speeds of pulse pressure tracings under different conditions of pressure and volume of flow. In making these, a Marey's tambour with rubber drumhead sufficiently heavy to withstand a high pressure, was connected by means of a T-tube to the outlet valve of the pump. The other end of the T-tube was connected to a



second T-tube, one end of which was attached to a mercury manometer and the other end to a safety valve made by dipping a glass tube to the required depth in a vessel of mercury contained in a large basin. The outflow of the pump then escaped through the column of mercury into the vessel containing it when the required pressure was reached, and flooding this, flowed into the basin. This safety valve also served as a means of producing capillary resistance artificially. The data obtained in making these tracings are shown in Table I.

TABLE I.  
 *$\frac{3}{4}$  Inch Bore Cylinder.*

	Record A.	Record B.
Systolic pressure, mm. Hg.....	142	150
Diastolic " " ".....	110	100
Pulse " " ".....	32	50
Strokes of pump per minute.....	96	96
Flow of fluid per minute, cc.....	55	127

The pulse pressures recorded in Table I are somewhat lower than others obtained without connecting in the tambour on account of the fact that the latter, being fairly large and elastic, acted as a reservoir and diminished the difference between the systolic and diastolic pressures.

TABLE II.  
*Details of Construction.*

Brass pump base.....	$8\frac{3}{16}$ " x 2" x $\frac{3}{4}$ "
" standard.....	7" high, of $\frac{3}{16}$ " brass.
Steel beam.....	$6\frac{1}{4}$ " x $\frac{1}{2}$ " x $\frac{1}{4}$ ", $3\frac{1}{2}$ " slit.
" spring arm.....	$3\frac{1}{2}$ " long.
Brass connecting rod.....	$5\frac{1}{2}$ " "
Hard rubber cylinders.....	$3\frac{3}{16}$ " "
" " pistons.....	1" "
Brass piston rods.....	$3\frac{11}{16}$ " "
Maximum stroke of piston.....	$\frac{3}{4}$ "
Minimum " " ".....	$\frac{1}{8}$ "
Cross head standard.....	$3\frac{5}{8}$ " long, over all.
Steel cam shaft.....	$5\frac{3}{8}$ " x $\frac{5}{16}$ "
" beam shaft.....	$4\frac{5}{8}$ " x $\frac{5}{16}$ "
Pulleys.....	$1\frac{3}{8}$ " and $1\frac{1}{2}$ " belt centers.
Center of cam shaft to center of beam shaft.	1" horizontal distance.
" " " " " " " " " " " "	$1\frac{5}{8}$ " perpendicular distance.

I have used the pump described in the foregoing pages in the perfusion of dog livers and kidneys. It is just as well adapted where a continuous flow is required, in the former case, as in the latter where a pulse pressure is necessary. In the former case the pump pulsations are obliterated by means of an air reservoir in the system. The only parts of the pump requiring much attention are the pistons and these must be freshly packed with thread occasionally. Vaseline is used as lubricant.

In concluding I wish to thank Mr. L. U. Boyle, the mechanic of the Medical School of the University of Minnesota, who made the pump, for helpful suggestions in carrying out the details.



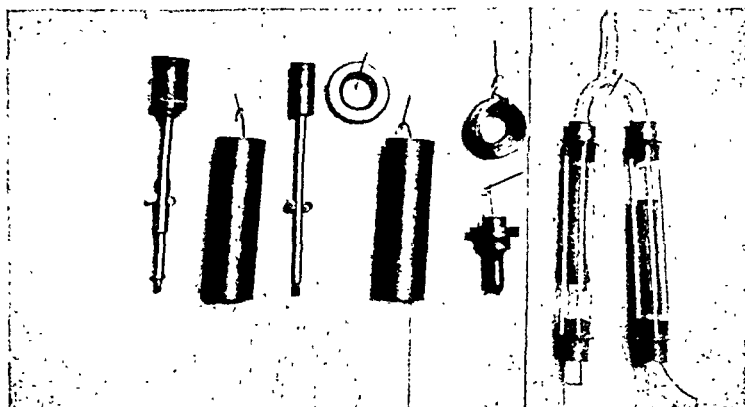
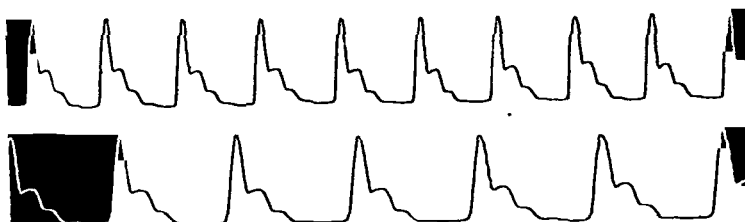
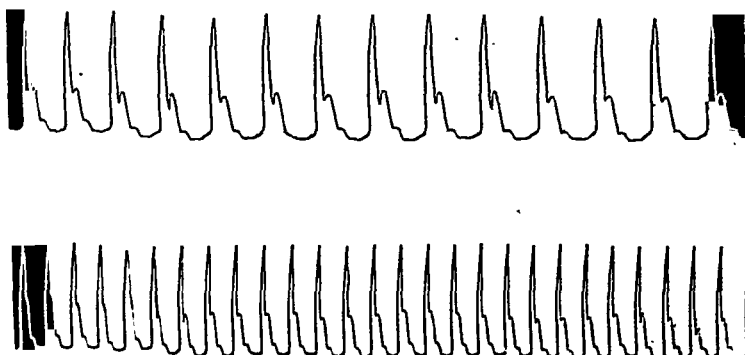


FIG. 1.



A.



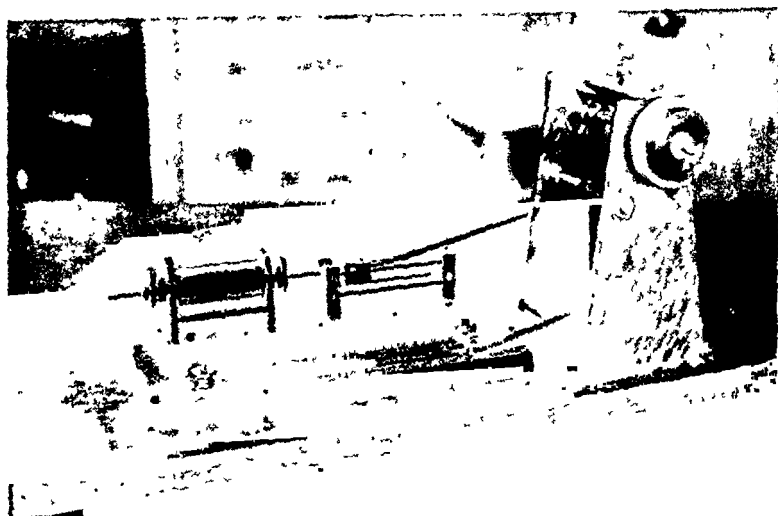


FIG. 3.

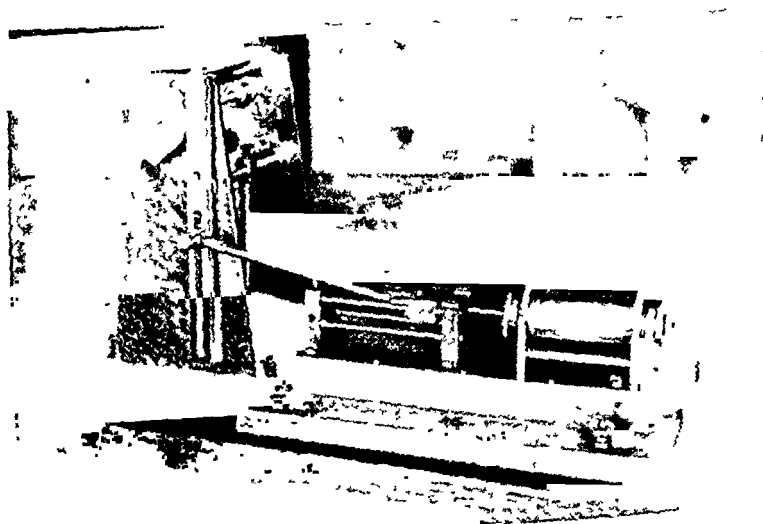


FIG. 4.

(Kingsbury: Perfusion Pump)

# THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

## IV. THE RATIO OF THE CONCENTRATION REQUIRED FOR THE ACCELERATING AND ANTAGONISTIC ACTION UPON THE DIFFUSION OF POTASSIUM SALTS.

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### I.

We have thus far considered only two critical phenomena in the effect of a second salt upon the rate of diffusion of KCl through the membrane of the *Fundulus* egg; namely, that a moderate concentration of the second salt accelerates, and a somewhat higher concentration retards the rate of diffusion of KCl. The retardation is the *antagonistic salt action*, while we called the accelerating effect upon the rate of diffusion the *salt effect*. In reality there are two more critical points. When we put washed eggs of *Fundulus* (*i.e.*, washed for 24 hours in  $H_2O$ ) into M/8 KCl solutions made up in various concentrations of a second salt (or of a balanced salt solution) we find that the retardation in the diffusion of KCl by a second salt, if plotted in a curve, frequently shows two maxima, *B* and *D* (Figs. 1 and 2), and three minima, *A*, *C*, and *E*. In these curves the logarithms of the concentration of the second salt (or mixture of salts) are represented as abscissæ, the percentage of hearts beating after from 4 to 6 days in each concentration as ordinates. The maxima, therefore, mean a retardation of the rate of diffusion of KCl (antagonistic salt action), the minima mean a rapid diffusion of KCl (general salt effect). *A* is the effect of a pure M/8 KCl solution (*i.e.*, an M/8 KCl solution made up in  $H_2O$ ). We find that the addition of a little salt retards the rate of diffusion (point *B*), that a

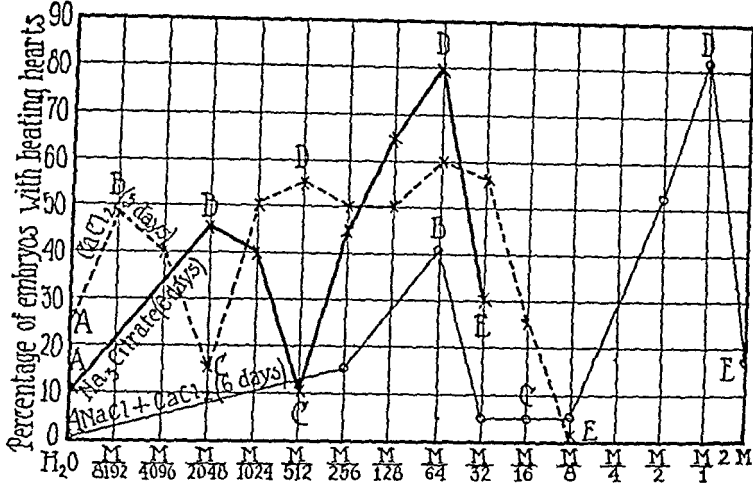


FIG. 1.

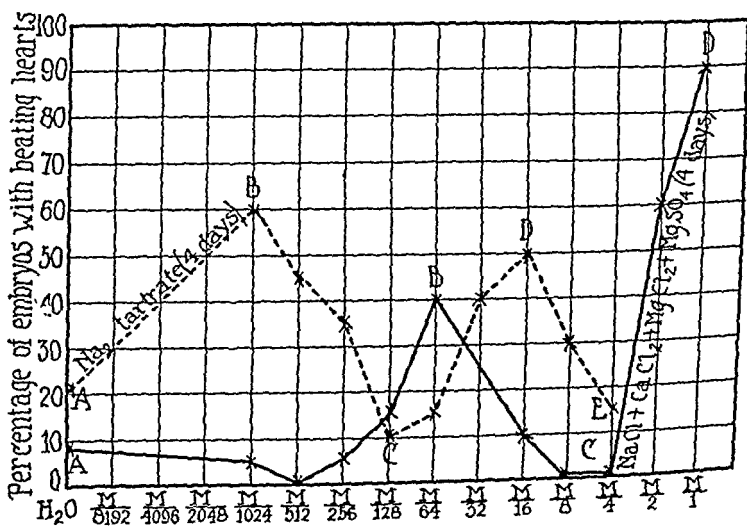


FIG. 2.

FIGS. 1 and 2. Retardation of the diffusion of  $M/8$  KCl into the egg by the addition of a second salt (or a mixture of salts) as shown after 4 to 6 days.  $M/8$  KCl was made up in  $H_2O$  and in increasing concentrations of the second salt. The logarithms of the concentrations of the second salt are on the axis of abscissæ, the ordinates give the percentage of hearts not yet poisoned. The curves show that a slight quantity of salt retards (point B), that a somewhat greater addition of salt accelerates (point C), and that a still greater concentration (point D) of salt retards considerably the diffusion of KCl into the egg. This latter effect is the antagonistic salt action. The retarding or antagonistic influence of a second salt upon the retardation of  $M/8$  KCl into the egg has therefore two maxima, a lower one B, and a higher one D. The latter is very constant while B may be lacking. The two summits are separated by a minimum representing the accelerating salt effect.

little more salt accelerates the rate of diffusion (the general salt effect, point *C*), and that a still higher concentration retards the diffusion again (point *D*), antagonistic salt action. A still higher concentration of the second salt *E* kills the eggs very rapidly, but this effect does not concern us here since it is plainly due to the diffusion of the second salt into the egg. The correctness of this view is demonstrated by the fact that the upper concentration at which the antagonistic effect ceases is lower for non-balanced solutions (*e.g.*, NaCl or NaNO<sub>3</sub>) than if these solutions are balanced through the addition of Ca or Mn. Both Ca and Mn retard the diffusion of the sodium salts into the egg, as shown in the first paper of this series. This effect of a high concentration is also observed if a non-electrolyte is added to the  $m/8$  KCl solution, while the peculiarities connected with lower concentrations of the second salt (the two antagonistic maxima separated by the accelerating minimum) are not observed in the case of the addition of non-electrolytes to a KCl solution.

Moreover, while an  $m/8$  KCl solution does not kill the embryo, but causes only a reversible cessation of heart beat, at the fourth critical point, *E*, the embryo is killed and undergoes coagulation. We may, therefore, omit this phenomenon from consideration in this paper, which deals only with the diffusion of potassium salts through the membrane.

As far as the influence of the second salt is concerned we therefore have to consider the following facts. The diffusion of an  $m/8$  KCl solution is retarded by a low concentration of a second salt (point *B* in the curve), accelerated by a somewhat higher concentration (salt effect, point *C*), and is considerably retarded by a still higher concentration of the second salt (antagonistic salt action, point *D* in the curve).

It may be well to point out a parallelism between these observations and the precipitation of globulins by salts, not because we have any reason to think of the existence of globulins in the membrane, but because it suggests the possibility that the elements of the membrane which determine the salt effect might be proteins. We have already mentioned the fact that Hardy's valence rule of the precipitation of proteins is applicable to the general salt effect; namely, that the efficiency of Cl : SO<sub>4</sub> : citrate is as 1 : 4 : 16. Concerning the globulins the following statement of Hardy is of interest.



Globulin solutions can be precipitated by neutral salts, and in this respect they exhibit very characteristic relations. Each salt acts as a precipitant at low concentration, and at high concentration. Between these two it acts as a solvent. The first precipitation occurs only when acid or alkaline globulin is present, and it is similar to the precipitation of hydrosols by small concentrations of salt in that the colloid particles are electrically charged. The second precipitation is a separation of solid globulin from a solution of salt globulin, and it is the precipitation of the colloid from a hydrosol in which the colloid particles are completely unchanged.<sup>1</sup>

The membrane of the egg of *Fundulus* is surely not a globulin, but the analogy in the general character of the curve in both cases is worthy of notice.

The analogy between our observations and the behavior of the globulins may be carried further. When we free the membranes of the eggs of *Fundulus* from salt at their external surface, by washing them in  $H_2O$  or in a solution of a non-electrolyte, KCl can diffuse through the membrane after enough of it has combined with the external surface to supply the salt effect necessary to make the diffusion possible. In the same way the diffusion of acid through the membranes of washed eggs can be explained (third paper of the series); namely, that acid can only diffuse through after having combined with a certain constituent of the membrane and having thus supplied the salt effect. According to our observations the efficiency of acid for the production of the salt effect is several hundred times greater than that of neutral salts. According to Hardy,<sup>2</sup> the solvent power of acids for globulins is from 200 to 500 times greater than that of neutral salts.

According to Sutherland,<sup>3</sup> the concentration of salts at which the precipitation of globulin just begins (*i.e.*, where the precipitating power just exceeds the solvent power of a salt) is about forty times that which produces complete solution of the original globulin (based on Mellanby's<sup>4</sup> observations). If we select the analogous points in the influence of the concentration of a second salt upon the diffusion of potassium salts through the membrane,

<sup>1</sup> Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 255.

<sup>2</sup> Hardy, *J. Physiol.*, 1905-06, xxxiii, 336.

<sup>3</sup> Sutherland, W., *Proc. Roy. Soc., Series B*, 1907, lxxix, 130.

<sup>4</sup> Mellanby, J., *J. Physiol.*, 1905-06, xxxiii, 338.

we should have to compare the concentration of maximal salt effect ( $C$  in our curves) in the diffusion of potassium with the concentration for maximal solution of globulin; and the concentration for beginning precipitation of globulin with the concentration of beginning antagonistic action to the diffusion of potassium (between  $C$  and  $D$  of our curve). While it is not too difficult to identify the point of maximal salt effect, the concentration for the beginning of the antagonistic action is difficult to establish. We must instead select another concentration; namely, that at which the antagonistic action is clearly established or a maximum, *i.e.*, point  $D$  of our curve. The two values, namely, that for maximal salt effect ( $C$ ) and the maximal antagonistic effect (point  $D$ ), were determined in the earlier experiments on the fish itself. With  $m/100$  KCl as the toxic concentration, the addition of less than eight or ten molecules of NaCl to one of KCl produced the maximal salt effect, and the addition of seventeen molecules of NaCl produced the marked antagonistic effect.<sup>5</sup> Hence the ratio of the two critical values of NaCl for the antagonistic effect and the maximal accelerating effect for the diffusion is as 17 : 8 or about 2 (or a little more).

We ascertained this ratio for the eggs of *Fundulus* in natural and artificial sea water and in different sodium salts to find out what the value would be and whether it would be constant for different salts. Eggs about a week old were washed for 24 hours in  $H_2O$  and then put into  $m/8$  KCl made up in  $H_2O$ , and in salt solutions of different concentrations. The concentrations of the various salts which showed an unmistakable accelerating and antagonistic effect were ascertained for each salt. It was necessary to wait for from 4 to 6 days to be sure that the correct values for the two effects were observed. Table I gives the results for various Na salts, alone and in balanced form.

This table allows us to compare the values for the "salt effect" (*i.e.*, the acceleration of the diffusion of KCl through the membrane) with the values for the antagonistic effect (*i.e.*, the inhibition of the diffusion). The former values in the table are in bold-faced type.

The ratio antagonistic effect: salt effect is given in Table II.

<sup>5</sup> Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxii, 155.

TABLE I.

After days		Percentage of washed eggs with beating hearts in m/8 KCl in												H <sub>2</sub> O
		M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	
6	Natural sea water...		20	0	0	10	15	15	25	20	5			5
6	NaCl + CaCl <sub>2</sub> + MgCl <sub>2</sub> + MgSO <sub>4</sub> ...	85	50	5	0	0	15	30	10	5	0	5		5
6	NaCl + CaCl <sub>2</sub> .....	83	55	0	5	5	5	43	5	15				0
6	NaCl.....	67	30	0	0	5	0	0	0	15				0
4	NaBr.....	50	80	15	0	0	20	20	20	45				45
6	NaNO <sub>3</sub> .....	0	36	5	5	15	16	4	30					10
6	NaNO <sub>3</sub> + MnCl <sub>2</sub> ...	70	33	0	0	33	30	30	25					10
4	Na acetate.....			40	75	59	15	45	10					45
4	Na <sub>2</sub> SO <sub>4</sub> .....			96	0	35	50	55						64*
6	Na <sub>2</sub> tartrate.....			0	10	30	35	5	0	10	25	25		0
6	Na <sub>3</sub> citrate.....						30	80	70	45	10	40	45	10

\* These eggs had been washed for 6 days in H<sub>2</sub>O.

TABLE II.

	Concentration giving salt effect.	Concentration giving antagonistic effect.	Ratio, antagonistic effect: salt effect.
NaCl.....	M/4	M/2 to M/1	About 2
NaNO <sub>3</sub> .....	M/4	M/2 to M/1	" 2
NaBr.....	M/4 to M/8	M/2	" 2
Na acetate.....	M/32	M/16	" 2
Na <sub>2</sub> tartrate.....	M/64	M/32	" 2
Na <sub>2</sub> SO <sub>4</sub> .....	M/8	M/4	" 2
Na <sub>3</sub> citrate.....	M/512	M/256 to M/128	" 2

For all the sodium salts the concentration required for the antagonistic effect is about twice as high as that required for the salt effect. These experiments allow only a rough approximation of the values, and it will be necessary to repeat them but they show that their value is almost identical with that found for the fish. The general order of relative efficiency for the salt effect is about the same as that found in the previous experiment with the exception of the value for Na<sub>2</sub>SO<sub>4</sub>, which is entirely different in this series. This difference may be due to the difference of conditions for these experiments in which the KCl solution and the solution of the second salt coexisted in the outside solution; whereas in the experiments mentioned in

the second paper only the second salt, and in the experiments in the third communication only the KCl existed in the outside solution of the eggs.

The analogy of these experiments with those on globulins is by no means complete. In the globulin experiments it is the same salt which in different concentrations causes solution and precipitation of the globulin. If the analogy were complete we should expect that KCl would prevent its own diffusion in a high enough concentration. The writer has in the few preliminary experiments not yet been able to find such a phenomenon, though a continued search may reveal it. For the prevention of diffusion of KCl a second salt seems to be required; while the salt effect can be supplied by either the potassium salt itself or by a second salt, *e.g.*, sodium salts. The fact that the potassium salt in a sufficiently high concentration does not supply its own antagonistic effect may, however, have another reason. The antagonistic effect (like the general salt effect) is based upon a reaction between the antagonistic salt and a constituent of the membrane (presumably one or more proteins), which requires time. Since it requires less time to produce the salt effect for the acceleration of the diffusion, it would follow that in a pure KCl solution of a very high concentration enough KCl would have time to diffuse through the membrane to cause cessation of the heart beat before the antagonistic effect in the membrane had time to establish itself.

## II.

Sometimes the salt effect is more easy of demonstration and sometimes the antagonistic effect, according to the method of experimentation. Eggs poisoned with KCl are not able to recuperate in solutions of non-electrolytes but will recuperate in the proper salt solutions. In this case we could only perceive an accelerating effect of the salts, but were not able to demonstrate an antagonistic effect by raising the concentration of the salt.

If, however, we try to poison the eggs with an  $M/8$  KCl solution and add a second salt we can demonstrate the acceleration of the diffusion into the egg (the general salt effect) in the case

In these experiments in which a second salt was always present with the KCl in the outside solution the antagonistic action could be more generally demonstrated than the accelerating effect. This is the reverse of what was found in the recovery experiments where only one salt was present in the outside solution and where the general salt action was clear but where no antagonistic action to KCl could be demonstrated.

## ELIMINATION OF MALATES AFTER SUBCUTANEOUS INJECTION OF SODIUM MALATE.\*†

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(Received for publication, October 22, 1916.)

Ohta<sup>1</sup> appears to be the only investigator who has studied the fate of malic acid in the animal body. He succeeded in isolating an appreciable quantity of malic acid from the urine of a dog which had received subcutaneous injections of sodium malate in large amounts. The acid thus isolated was fully identified by its melting point, its optical rotation, and by means of an elementary analysis. Ohta also described in some detail experiments on a few rabbits and dogs which had received fairly large quantities of sodium malate *per os* and by subcutaneous injection. By determining the percentage of malic acid in the urine of his experimental animals, Ohta concluded that in some cases malates escaped complete oxidation in the body. The percentage elimination was, however, always small, never exceeding 5 per cent of the amount given by mouth, and never more than 6 per cent of the amount injected subcutaneously.

The present paper gives the results of a comparative study of the elimination of malates following subcutaneous injection of sodium malate in rabbits and in cats. In view of the incompleteness of Ohta's protocols and the meagerness of his analytical data, some of his experiments have been repeated.

\* Published with the permission of the Secretary of Agriculture.

† This problem was suggested by Dr. William Salant, Pharmacologist in charge of the Pharmacological Laboratory, Bureau of Chemistry, United States Department of Agriculture.

<sup>1</sup> Ohta, K., *Biochem. Z.*, 1912, xliv, 482.

## EXPERIMENTAL.

*Analytical Procedure.*

Repetition of Ohta's work led to an examination of his method for the determination of malic acid in urine. His procedure involves a modification of Yoder's polariscopic method<sup>2</sup> for the determination of malic acid in maple products. This method depends on the enhancement of the levorotation of malic acid solutions caused by the addition of uranyl acetate.

In his preliminary experiments, which need not be given in detail, Ohta used 5 cc. of a saturated aqueous solution of uranyl acetate for every 10 cc. of normal<sup>3</sup> urine to which known quantities of malic acid had been added. He assumed that the uranyl acetate was then present in excess, and after filtering his mixture, polarized the clear yellow filtrate and calculated the amount of malic acid by the use of Yoder's empirical formula. The fact that he could recover 93 per cent of the malic acid added seemed to show that his technique was correct, and the procedure was followed in determining the amount of malic acid eliminated by his experimental animals.

In a series of experiments in which Ohta's method was used on normal urines containing known amounts of malic acid, the author was in some cases able to recover 88 per cent of the malic acid present. In other urines, however, the filtrate after uranyl acetate addition was optically inactive, and it was impossible to show even the presence of malates. It soon became evident that the success in following Ohta's procedure depended on the nature of the individual urines examined. In some cases phosphates or other urinary constituents caused complete precipitation of the uranium reagent, leaving an optically inactive filtrate, and thus invalidated the test. It was found necessary, therefore, to devise a modification which would insure a true excess of uranyl acetate in the filtrate used for polarization.

The following analytical procedure, involving a slight but essential modification of Ohta's method, was found satisfactory for the determination of small amounts of malate in urine. 10 cc. of urine,<sup>4</sup> neutralized (litmus) with normal NaOH or with dilute acetic acid, depending on the initial reaction to litmus, were treated with 2 cc. of a 30 per cent acetic acid solution and

<sup>2</sup> Yoder, P. A., *J. Ind. and Eng. Chem.*, 1911, iii, 569.

<sup>3</sup> Malate-free urine, which after neutralization with NaOH was acidified with varying amounts of acetic acid.

<sup>4</sup> If the urine is alkaline and contains a sediment, it is best first to render the mixture acid with hydrochloric acid, filter, and exactly neutralize the filtrate with NaOH.

diluted with water to 25 cc. (or to some other definite convenient volume). Powdered uranyl acetate was added *in large excess*, the mixture boiled for about 2 minutes, and vigorously shaken. After heating, *solid* uranyl acetate still remained in contact with the solution. Upon cooling to room temperature, the solution was again diluted to 25 cc. (or to the volume which obtained prior to heating), and the mixture allowed to stand no less than 5 hours in a dark cupboard. At the end of this period the solution was filtered into a 200 mm. or a 100 mm. observation tube and polarized at about 20°, using white light and the bichromate cell. The Bates saccharimeter was used in all determinations, the scale reading in degrees Ventzke. The percentage of malic acid present in the solution was found from data tabulated by Gore.<sup>5</sup> By a simple proportion the amount of malic acid in the initial volume of solution was computed and the weight of sodium malate estimated.

Table I shows with what success the above method may be applied and also indicates the limitations of the method.

TABLE I.

*Results of Preliminary Experiments on a Method for the Determination of Malic Acid in Urine.*

Urine (normal).	Malic acid added to 10 cc. of urine.	Malic acid recovered.
	<i>mg.</i>	<i>mg.</i>
Human.....	12.5	14.4
“.....	25	24
“.....	50	49.8
“.....	125	118
“.....	125	116
“.....	100	94
Rabbit.....	37.5	37.2
“.....	50	50.6
“.....	50	49.2
“.....	100	93.8
“.....	None.	None.
Cat.....	100	95
“.....	100	94.6

<sup>5</sup> Gore, H. C., Report on Fruit and Fruit Products, *J. Assn. Off. Agric. Chem.*, 1915, i, 481.



Experiments have shown that the above procedure may be carried out with slight modification in the presence of glucose. In glycosuria, it is essential to make a polarimetric reading<sup>6</sup> prior to the addition of uranyl acetate, and to make allowance for the dextrorotation by adding it to the levo reading obtained after addition of the uranium reagent.

### *Animal Experiments.*

Aqueous solutions used for injection ordinarily contained 100 gm. of anhydrous sodium malate per liter of solution, and are referred to in the protocols as 10 per cent solutions. When occasional large injections were necessary, 25 per cent solutions were used. Urines of the experimental animals were preserved with toluene and were analyzed as soon as possible after collection. In each experiment a convenient aliquot portion of the total urine voided during a 24 or 48 hour pre-observational period was polariscopically examined according to the above procedure. In a few cases such urines showed a negligible optical activity; ordinarily they were optically inactive. Aliquot portions of the urine eliminated during the 22 to 24 hour period following injection were examined and their malate content was determined. The urine of a second 24 hour period was also analyzed. In most cases all of the undestroyed malic acid (found in the urine) was eliminated in the course of the first 24 hours following malate injection. In some individuals, however, small but appreciable quantities of malate could be recovered from the urine the 2nd day following injection. In such cases the amount eliminated was so small that further search for malate was ordinarily deemed unnecessary.

### *Experiments with Oat-Fed Rabbits.*

Injection of sodium malate was in all cases followed by marked elimination of malates in the urine. In two cases, 3.3 gm. of sodium malate per kilo of body weight were injected, but as one of our experimental animals (Rabbit 1) died within 48 hours after such an injection, the dose was decreased to 1 gm. per kilo in our other experiments. The protocol of Rabbit 1 follows. It should be noted that the percentage elimination of malate is in

<sup>6</sup>The error in glucose reading, owing to the very slight levorotation of the malic acid, is negligible.

## PROTOCOL I.

*Subcutaneous Injection of Oat-Fed Rabbit 1 with 3.3 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Urine eliminated.	Data relating to malate elimination in urine.
Oct. 30 Nov. 1	9.00 a.m.	Weight 1.705 kg. 48 hr. urine collected.	80 cc.	Urine was optically inactive after uranyl acetate addition. <i>Malates and other optically active substances absent.</i>
	11.20 "	Injected subcutaneously (two sites) 22.5 cc. of a 25 per cent sodium malate solution ( <i>i.e.</i> , 5.63 gm. of sodium malate).		
	11.50 "	No symptoms noted.		
	1.30 p.m.	No symptoms other than diarrhea.		
	4.30 "	No further symptoms noted.		
" 2	9.00 a.m.	Muscular incoordination and tremors noted. Marked weakness of the extremities.		Dextrorotatory prior to uranyl acetate addition. Total elimination corresponds to 970 mg. of sodium malate (due correction being made for glucose) = 17 per cent of the total malate injected.
	9.30 "	24.5 hr. urine collected.	100 cc. Urine alkaline. <i>Glycosuria.</i>	
" 3	9.00 "	Rabbit found dead.		

## PROTOCOL II.

*Subcutaneous Injection of Oat-Fed Rabbit 2 with 1 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Rations.		Urine eliminated.	Data relating to malate elimination in urine.
			Food (oats).	Water.		
			gm.	cc.		
Nov. 6	Noon.	Weight 1.96 kg.				
" 8	9.00 a.m.	45 hr. urine collected.	Not recorded.	Not recorded.	98 cc. of faintly acid urine. Albumin test+; slight glycosuria.	Urine was optically inactive after uranyl acetate addition. Malates absent.
	10.01 "	Injected subcutaneously 19.5 cc. of a 10 per cent sodium malate solution.				
	11.10 "	No symptoms noted.				
	12.30 p.m.	No symptoms noted.				
	3.00 "	No symptoms noted.				
9	9.00 a.m.	Animal apparently normal.	80	200	88 cc. of urine collected. Albumin, heavy trace; sugar, trace.	Total elimination of malate corresponds to 321 mg. of sodium malate = 16.5 per cent of the total amount injected. Malates absent.
" 10	9.00 "	Animal apparently normal.	75	120	59 cc.	

this case a *minimal value*, owing to the lack of a second 24 hour sample. Protocol II outlines a typical experiment with an oat-fed rabbit which received a subcutaneous injection of 1 gm. of sodium malate per kilo.

It is needless to give further protocols. The results of experiments on oat-fed rabbits are summarized in Table II.

TABLE II.  
*Summary of Experiments on Oat-Fed Rabbits.*

Rabbit.	Sex.	Description.	Weight.	Sodium malate injected per kg. of body weight.	Sodium malate injected.	Sodium malate eliminated.	
						gm.	per cent
1	♂	White	1.705	3.3	5.63	0.97	17.0
2	♂	Belgian.	1.96	1.0	1.95	0.32	16.5
3	♂	White.	1.985	3.3	6.63	1.42	21.5
4	♀	Belgian.	1.85	1.0	1.85	0.06	3.0
5	♂	"	1.975	1.0	2.00	0.17	8.5
6	♂	"	2.435	0.94	2.30	0.247	10.5
7	♂	"	2.35	0.94	2.21	0.447	20.0
Average .....			.....	.....	.....	.....	14.0

*Experiments with Carrot-Fed Rabbits.*

In these experiments the large volumes of urine eliminated, caused by the carrot diet, made it necessary to concentrate the urine by evaporation. Aliquot portions of these concentrated urines were used for malate determinations. Difficulty was experienced in obtaining concordant polarimetric readings, owing to the dark color of the concentrates; hence the results given in Table III must be regarded as merely approximate. Since, however, these results are all of the same order of magnitude as those obtained in the case of oat-fed rabbits, it is apparent that the change in diet from oats to carrots is without effect on the elimination of malate.

*Experiments with Meat-Fed Cats.*

The experimental animals had previously been used in a series of experiments with sodium citrate. After recovering fully from the effects of citrate injection, the animals were kept under

TABLE III.  
Summary of Experiments on Carrot-Fed Rabbits.

Rabbit.	Sex.	Description.	Weight.	Sodium malate injected per kg. of body weight.	Sodium malate injected.	Sodium malate eliminated.	
						gm.	per cent
8	♀	Maltese.	2.075	1.0	2.08	0.24	11.5
9	♀	"	2.33	1.0	2.35	0.38	16.0
10	♂	White.	1.445	1.0	1.45	0.26	18.0
11	♂	Belgian.	2.335	1.0	2.35	0.25	10.5
Average..							14.0

observation in separate cages for a month or more. The cats were in good condition and ate normally at the outset of the present experiments.

A preliminary trial, in which doses of 3.3 gm. of sodium malate per kilo of body weight were subcutaneously injected into two cats, resulted in the death of one animal, whose urine during the observational period was not examined. In consequence the dose was decreased to 1 gm. per kilo. In general, these smaller doses caused no symptoms or, at most, a slight general depression. Malates were invariably found in the urine after injection, as shown in Table IV.

TABLE IV.  
Summary of Experiments on Cats.

Cat.	Sex.	Description.	Weight.	Dose per kg.	Sodium malate injected.	Sodium malate eliminated.	
						gm.	per cent
1	♀	White and tiger.	2.4	1.0	2.4	0.90	37.5
2	♂	Black and white.	2.4	1.0	2.4	0.41	17.0
3	♀	Black.	2.385	1.0	2.4	1.00	41.5
4	♂	Gray and white.	1.83	1.0	1.8	0.31	17.5
5	♀	White and tiger.	2.45	0.94	2.3	0.44	19.0
6	♂	" " "	1.98	3.3	6.63	1.9	29.0
6*	♂		2.12	0.94	1.97	0.42	21.0
Average..							26.0

\* Reinjected after making good recovery, 2 weeks after the first injection.

Protocols III and IV are typical.

*Subcutaneous Injection of Cat 2\* with 1 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Rations.		Urine voided.	Data relating to malate elimination in urine.
			Meat.	Water.		
Nov. 15	9.00 a.m.	Condition of animal good. 48 hr. urine collected.	gm. 100	cc. 40	76 cc. Faint trace of albumin. No glycosuria.	Urine inactive after addition of uranyl acetate. <i>Malates absent.</i>
	10.50 "	Injected subcutaneously 24 cc. of a 10 per cent sodium malate solution.				
	1.05 p.m.	No symptoms noted.				
	2.50 "	No symptoms noted.				
	4.25 "	No symptoms noted.				
" 16	9.00 a.m.	Animal apparently normal.	100	80	56 cc. Free from albumin and sugar.	Test for malates made on 10 cc. of urine showed rotation of $-3.00^{\circ}$ V. in a 100 mm. tube. This corresponds to a total elimination of 410 mg. of sodium malate; i.e., a 17 per cent elimination.
" 17	9.00 a.m.		100	30	56 cc.	Test on 10 cc. of urine showed rotation of $-0.35^{\circ}$ V. Total elimination of 50 mg. of sodium malate; i.e., 2 per cent.
" 18	9.00 "		100	20	58 cc.	<i>Malates absent.</i>

\* Received 1 gm. of sodium citrate per kg. subcutaneously, on Oct. 4, 1915. Weight of animal on Nov. 13, 1915, 2.4 kg.

PROTOCOL IV.

*Subcutaneous Injection of Cal 1\* with 1 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Rations.		Urine voided.	Data relating to malate elimination in urine.
			Meat.	Water.		
			gm.	cc.		
Nov. 13	9.00 a.m.	Weight 2.4 kg. Condition good.				
" 15	9.00 "	48 hr. urine collected (49 hr. rations).	100	60	102 cc. containing neither albumin nor sugar.	Test for malates on 10 cc. of urine negative.
	10.55 "	Injected subcutaneously 24 cc. of a 10 per cent sodium malate solution.				
	1.05 p.m.	No symptoms noted.				
	2.50 "	No symptoms noted.				
	4.25 "	No symptoms noted.				
" 16	9.00 a.m.	Animal apparently normal.	100	90	105 cc. No albumin and no sugar.	Test on 10 cc. of urine shows a rotation of $-3.55^{\circ}$ V. in 100 mm. tube. This reading corresponds to a total elimination of 900 mg. of sodium malate; i.e., 37.5 per cent elimination.
" 17	9.00 "	Animal apparently normal.	100	20	85 cc.	Test for malates on 10 cc. of urine negative.

\* Received 1 gm. of sodium citrate per kg. subcutaneously, on Oct. 7.

## DISCUSSION.

As shown by the results of preliminary experiments, the quantity of uranyl acetate necessary to permit the quantitative estimation of malates in the urine of certain individual animals is much greater than the amounts used by Ohta in his work. It appeared possible therefore that Ohta himself did not in every case secure a quantitative determination of the malates present in the urine. As a matter of fact, the analytical figures given above indicate strongly that his data were subject to error. His general conclusion that *malates are incompletely destroyed in the body* is substantiated by the figures given above, and indeed more strikingly than by his own. Whereas Ohta was never able to recover from the urine more than 6 per cent of the amount of malate injected into rabbits, and was in the case of one urine unable to demonstrate the presence of malates, the results of the present paper show an elimination of from 3 to 21 per cent of the total malate injected. Comparative data obtained with oat-fed rabbits on the one hand, and carrot-fed rabbits on the other, indicate that the effects of diet on malate elimination are negligible in the case of these animals. Without wishing to overemphasize an average of results which themselves cover a rather wide range, it may be well to point out that rabbits eliminate, on the average, 14 per cent of the malate injected, whereas the average elimination in cats is 26 per cent.

The lowest elimination in the case of rabbits was 3 per cent; the highest was roughly 21 per cent. The lowest malate elimination in the case of cats was about 17 per cent, the highest exceeded 41 per cent of the amount injected. A real difference therefore appears to exist between cats and rabbits in their ability to destroy malates after subcutaneous injection.

## SUMMARY AND CONCLUSIONS.

1. The estimation of small amounts of malic acid in urine was satisfactorily accomplished by using a modified Ohta-Yoder method. The essential modification consisted in *saturating* the urine with *powdered* uranyl acetate, instead of adding a *saturated solution* of the reagent.

2. Sodium malate, injected subcutaneously in moderate doses,



was shown to be incompletely destroyed by rabbits and cats, and was eliminated in the urine. Rabbits eliminated from 3 to 21 per cent, whereas cats eliminated from 17 to 41.5 per cent of the total amount of malate injected.

3. Elimination of undestroyed malate in the urine of the experimental animals was usually complete within 24 hours after injection.

4. Subcutaneous injections of sodium malate in amounts *not exceeding* 1 gm. per kilo were *not* followed by nephritis or glycosuria.

5. Rabbits showed no toxic symptoms after receiving subcutaneous injections of sodium malate in amounts *not exceeding* 1 gm. per kilo. Subcutaneous injections of corresponding amounts of sodium malate into cats caused either no symptoms or at most a slight temporary depression.

6. Toxic symptoms in both cats and rabbits followed the injection of larger amounts (3.3 gm. per kilo) of sodium malate. No effort, however, has been made to fix the lowest limit of the toxicity of sodium malate.

# THE RELATION BETWEEN THE WATER AND GLUCOSE CONCENTRATION OF THE BLOOD.

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Polyuria, edema, and desiccation are all characteristic manifestations of diabetes mellitus. These changes in the water metabolism are generally regarded as the result of glycosuria and the increase of glucose in the fluids and tissues of the body. All attempts to associate hydremic or inspissated blood with a parallel increase or diminution of the blood sugar have thus far failed, and Morawitz,<sup>1</sup> in summarizing the situation, shows how the blood of the diabetic has been found by some investigators to be inspissated, while others have demonstrated a hydremia. From the therapeutic point of view, these questions are of great importance, for on their solution depends the regulation of the diabetic's water intake. Applying micro methods to this problem, it was possible to obtain the blood in 10 to 15 minute intervals, determine the sugar and water content in each specimen, and note the relation between the two. Where it was desired to raise the sugar content of the blood, glucose was given by mouth.

The method of Lewis and Benedict<sup>2</sup> was used to determine the blood sugar. The water content of the blood was ascertained by putting about 1 cc. of freshly drawn blood into a tin capsule of known weight, weighing immediately to determine the quantity of blood, placing the capsule, after removal of the cover, in a drying oven at 98°C. for 24 hours, then allowing it to remain in a desiccator for 48 hours, or until a constant weight was established; after weighing again, the percentage of water lost could be calculated. This method has errors which are apparent. However, it yields results which are comparative, the error being constant. Good duplicates are obtained with this procedure, as may be seen in Table I.

<sup>1</sup> Morawitz, P., Oppenheimer's Handb. Biochem. Menschen u. Thiere, Jena, 1910, iv, 251. The bibliography of the subject is detailed here.

<sup>2</sup> Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

TABLE I.

*Results of Duplicate Analyses of the Water Content of the Blood by the Authors' Method.*

Blood specimen No.	Percentage of water.	
1	79.3	79.4
2	80.4	80.4
3	80.6	80.6
4	80.8	80.8
5	80.7	80.7
6	80.7	80.7

In order to save space, only the essential protocols will be reported.<sup>3</sup>

*The Control Cases.*—In these individuals, no attempt was made to raise or lower the blood sugar. Two types of blood sugar curves are readily distinguished (Table II), one in which the glycemia constantly diminishes, presumably returning to a normal level after a meal (R. M. and S. R., Table II), the other in which the blood sugar remains constant (J. C., Table II). Considering the short intervals in which the observations were made, it is rather surprising to find such a marked variation in the water content of the blood in each individual. The difference between the highest and the lowest reading in each case is as follows: P. M., 1.3 per cent, S. R., 1.3 per cent, J. C., 0.9 per cent. The variations, moreover, did not follow the falling blood sugar curve seen in R. M. and S. R., but ran an entirely independent course, nor did they remain constant in J. C., as the blood sugar did. Hence it is possible to conclude that spontaneous fluctuations in the amount of blood sugar, as they occur in the non-diabetic individual, are not paralleled by changes in the water content of the blood.

*Normal Individuals Receiving 100 Gm. of Glucose.*—The next step was to study the effect of the ingestion of 100 gm. of glucose. Although this may not raise the blood sugar to any marked degree, it may possibly be responsible for an increased hydremia, by influencing the water metabolism in other ways. In no instance

<sup>3</sup> We wish to acknowledge the cooperation of Drs. L. Hamman and I. Hirschman in furnishing many of the blood sugar determinations.

TABLE II.  
*Data of Control Cases.*

Case.	Time.	Blood.	
		Glucose.	Water.
	<i>p.m.</i>	<i>per cent</i>	<i>per cent</i>
R. M. Hypertrophic arthritis of lumbar spine.	2.40	0.129	79.4
	2.57 17 min. interval.		79.0
	3.15 18 " "	0.115	80.3
	3.45 30 " "	0.105	79.9
	4.10 25 " "	0.102	80.3
	4.30 20 " "	0.099	79.9
S. R. Chronic endocarditis. Mitral insufficiency. No symptoms of cardiac decompensation.	2.25	0.138	79.4
	2.38 13 min. interval.	0.129	80.4
	2.54 16 " "	0.112	80.6
	3.12 18 " "	0.102	80.8
	3.27 15 " "	0.100	80.7
	3.44 17 " "	0.093	80.7
J. C. Neurasthenia.	2.43	0.096	78.4
	2.55 12 min. interval.	0.096	78.3
	3.10 15 " "	0.096	78.1
	3.25 15 " "	0.096	78.7
	3.38 13 " "	0.096	79.0
	3.50 12 " "	0.095	78.9

did this occur. This is contrary to the findings of Fisher and Wishart,<sup>4</sup> who demonstrated a hydremic plethora in dogs 2 hours after the ingestion of glucose. These authorities base their conclusions on variations in the hemoglobin content of the blood. It may be that the proportionately greater dose of glucose in relation to the body weight (50 gm. per dog) was responsible for the differences obtained in these observations, as compared to ours. The rise from 76.6 to 78.9 per cent of water in H. (Table III) 3 minutes after the grape sugar solution was swallowed can scarcely be the result of the absorption of dextrose in so short a time. Neither G. nor two other normal individuals (not charted) gave any indication of such an influence. In H. the variation between the maximum and minimum water percentage was as high as 2.3 per cent; in the other individuals it was 0.8, 0.9, and

<sup>4</sup> Fisher, G., and Wishart, M. B., *J. Biol. Chem.*, 1912-13, xiii, 49.

1.2 per cent, figures comparable to those obtained in the control cases (Table II). What caused the one very high figure for hydremia in H. is an open question. In G. a large volume of urine was put out constantly during the period of observation. This apparently did not affect the water concentration of the blood.

The exhibition of 100 gm. of glucose in normal individuals has no effect in changing the concentration of the blood. Polyuria observed during the course of 3 hours does not necessarily result in variations of the water content of the blood.

TABLE III.  
*Data on Normal Individuals Receiving 100 Gm. of Glucose.*

Case.	Time.	Blood.		Urine volume per hr.
		Glucose.	Water.	
		per cent	per cent	cc.
H. Normal.	8.45 a.m.	0.090	76.6	
	8.47 " 100 gm. of glucose in 300 cc. of water.			
	9.00 " 15 min. interval.	0.085	78.9	7
	9.15 " 15 " "	0.124	76.7	25
	9.31 " 16 " "	0.120	77.9	64
	9.45 " 14 " "	0.099	77.7	96
	10.00 " 15 " "	0.080	77.4	72
	10.30 " 30 " "	0.072	77.6	52
	11.01 " 31 " "	0.062	77.4	69
	12.03 p.m. 62 " "	0.077	76.6	130
G. Normal.	8.35 a.m.	0.109	79.2	120
	8.42 " 100 gm. of glucose in 300 cc. of water.			
	8.55 " 20 min. interval.	0.112	78.9	215
	9.10 " 15 " "	0.132	79.3	137
	9.30 " 20 " "	0.137	79.6	320
	9.49 " 19 " "	0.111	78.6	215
	10.27 " 38 " "	0.103	78.8	160
	11.28 " 61 " "	0.109	79.1	135

*Cases of Diabetes Mellitus Receiving 100 Gm. of Glucose.*—A few of these cases are detailed in Table IV. In patients E. and C. hydremia and glycemia show no parallel whatever. In fact, the water concentration of the blood runs a course much as it does in

TABLE IV.

*Relation between Water Concentration and Glucose of the Blood in Diabetics in Whom a Hyperglycemia Is Produced by the Administration of Glucose.*

Case	Time	Blood		Urine volume per hr.
		Glucose	Water	
		per cent	per cent	cc
R. Diabetes mellitus.	8 45 a.m.	0 137	78 9	76
	8 46 " 100 gm. of glucose in 300 cc. of water.			
	9 10 " 25 min. interval.	0 171	78 9	205
	9 25 " 15 " "	0 200	78 8	77
	10 07 " 42 " "	0 240	79 2	50
	11 07 " 60 " "	0 238	79 6	88
	11 36 "			83
	12 10 p.m. 63 " "	0 123	79 3	48
B. Diabetes mellitus.	8 52 a.m.	0 070	79 6	172
	8 55 " 40 gm. of glucose in 300 cc. of water.			
	9 12 " 20 min. interval.	0 090	78 8	117
	9 25 " 13 " "	0 186	79 5	134
	9 40 " 100 cc. of water.			
	9 43 " 18 min. interval.	0 210	78 0	312
	10 32 " 49 " "	0 218	77.2	380
	10 40 " 100 cc. of water.			
E. Diabetes mellitus	11 30 " 58 min. interval.	0 210	77 8	34
	9 20 a.m.	0 108	79 7	
	9 25 " 100 gm. of glucose in 300 cc. of water.			
	9 45 " 25 min. interval.	0 126	79 3	138
	10 45 " 60 " "	0 171	78 9	
	10 58 "			895
	11 55 " 70 " "	0 173	79 6	
	12 20 p.m.			365
C. Diabetes mellitus.	12 45 " 50 " "	0.126	79 7	105
	8 45 a.m.	0 091	78 1	142
	8 48 " 100 gm. of glucose in 300 cc. of water.			
	9 10 " 25 min. interval.	0 124	77 6	122
	9 30 " 20 " "	0 210		110
	9 50 " 20 " "	0 190	78 3	83
	10 25 " 35 " "	0 174	78 6	94
	11 07 " 42 " "	0 155	78 6	102
	12 00 n. 53 " "	0 094	78 0	52

TABLE IV—*Concluded.*

Case.	Time.	Blood.		Urine volume per hr.  cc.
		Glucose.  per cent	Water.  per cent	
D. Diabetes mellitus.	Mar. 1 Glycemia as ob-	0.356	79.0	
	" 15 served in the	0.146	79.4	
	" 17 course of the disease. No glu- cose adminis- tered.	0.134	79.7	

the control individuals in whom no hyperglycemia was produced. How independent these two factors are of one another is seen in the last case, D., of Table IV, where a glycemia of 0.356 per cent was accompanied by a lower hydremia than a blood sugar of 0.134 per cent. In a very few cases (R., Table IV), a suggestive parallelism may be observed between the water and glucose content of the blood; in others (B., Table IV), an apparent effect of polyuria in inspissating the blood is manifested. Various authors have noted a tendency on the part of the blood and tissues to lose water<sup>5</sup> during the final stages of diabetes. It is probable that the excessive polyuria in such cases is the controlling factor.

#### CONCLUSIONS.

The water concentration of the blood in the normal individual often shows a variation of about 1 per cent during the course of a few minutes. There is no assignable cause for this inconstancy. Raising or lowering the sugar content of the blood does not give rise to a corresponding change in its concentration. If glycemia plays a part in controlling the water content of the blood, this is so slight in its effect that other factors, such as polyuria, etc., nullify its results, except in rare instances.

<sup>5</sup> Magnus-Levy, A., *Arch. exp. Path. u. Pharm.*, 1901, xlv, 389. Rumpf, T., *Z. klin. Med.*, 1902, xlv, 260. Dennstedt, M., and Rumpf, T., *Z. klin. Med.*, 1906, lviii, 84. Von Noorden, C., *Metabolism and Practical Medicine*, Chicago, 1907, iii, 614.

# THE FOLIN AND DENIS METHOD OF NITROGEN DETERMINATIONS BY DIRECT NESSLERIZATION, AND ITS APPLICATION TO SPINAL FLUIDS.

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Folin and Denis<sup>1</sup> have recently modified the micro method for nitrogen determinations, by substituting phosphoric acid for part of the sulfuric acid ordinarily employed in the destructive digestion of the nitrogenous material and by reducing the amount of free alkali in Nessler's reagent. These changes have enabled them to Nesslerize directly the ammonia produced during the digestive process, thus eliminating the aeration procedure connected with these determinations.

In attempting to apply this method to nitrogen determinations in spinal fluids, a serious difficulty has been encountered. Folin and Denis have already directed attention to the turbidity of the Nesslerized solutions due to the presence of silicates formed by the action of phosphoric acid and heat on the glass during the destructive digestion. They advise the removal of these silicates either by centrifugation or by filtration through a small cotton plug and state, "if the sediment obtained is mixed with a red deposit, the Nesslerization has not been successful and the determination must be discarded."

The difficulty just discussed has actually presented itself in this laboratory in every determination carried out according to the original directions. Furthermore, the standard solution also invariably showed clouding, indicating that the turbidity is not due to the silicates alone. In these trials, the concentrated acid mixture employed consisted of three volumes of concentrated phosphoric acid, one volume of concentrated sulfuric acid, and one-fifteenth volume of 10 per cent copper sulfate solution. The two acids were first mixed and allowed to stand over night

<sup>1</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.



to facilitate the precipitation of calcium. The mixture was then filtered by means of a Buchner funnel, through a dry mat of asbestos, after which the copper sulfate solution was added.

15 cc. of 10 per cent sodium hydroxide were found to be sufficient to neutralize 1 cc. of the concentrated acid mixture and permit a surplus of 2 cc. of alkali in the solution. This was determined in the following manner. 1 cc. of the concentrated acid mixture on titration with 10 per cent sodium hydroxide, with phenolphthalein as an indicator, required 9.5 cc. of alkali for neutralization. About three-fourths of this acidity (or 7.1 cc.) was due to phosphoric acid. As pointed out by Folin and Denis, one-third of the base combining power of the phosphoric acid is not included in the titration value. Therefore, 9.5 cc. + 3.5 cc., or 13 cc., were required for the neutralization of the acid. In order to permit a surplus of 2 cc. of 10 per cent sodium hydroxide, 15 cc. of the alkali were employed.

Nessler solution was prepared precisely as directed by Folin and Denis.

An attempt to ascertain the nature of the precipitate responsible for the turbidity in the standard disclosed by physical and chemical examination the probable presence of calcium. The precipitate of calcium formed on mixing the concentrated phosphoric and sulfuric acids, as stated above, is filtered off through a mat of asbestos. Traces of calcium, however, are soluble in an acid solution. When, therefore, the solution is rendered alkaline by the addition of 10 per cent sodium hydroxide, these traces separate out and cause turbidity on Nesslerization.

Having thus been unable to prepare clear solutions for color comparisons, an attempt was made to *filter off the precipitate before Nesslerization*. The results were entirely satisfactory. Nessler solution was thus added to water-clear filtrates in each case and clear solutions for color comparisons were obtained without exception.<sup>2</sup>

<sup>2</sup> It is likely that due to a higher purity of chemicals employed in the laboratory of Folin and Denis, no precipitation occurred on addition of the 10 per cent alkali. However, as is indicated by their statement quoted above, apparently some determinations have to be discarded from time to time on account of the red deposit mixed with the precipitate. With the modification suggested, no determination need ever be discarded, as is pointed out in the text later.

The probable loss of ammonia during the filtration process early suggested itself. On addition of a sufficient quantity of 10 per cent sodium hydroxide to neutralize the concentrated acid and permit a surplus of 2 cc. of alkali, ammonia is set free in the solution. Inasmuch as ammonia is extremely volatile, it was feared that traces of it might be lost during the filtration process. It was found, however, that owing to the extreme solubility of ammonia, significant losses do not occur. This was determined in the following manner.

To a 100 cc. volumetric flask, A, were added 1 cc. of the concentrated acid mixture, 15 cc. of alkali, and 20 cc. of the standard ammonium sulfate solution containing 1 mg. of nitrogen, made up to the mark, shaken, and filtered. 50 cc. of the water-clear filtrate were measured out into another 100 cc. volumetric flask, 10 cc. of Nessler solution added, made up to the mark, and shaken. To a volumetric flask, B, were added 1 cc. of concentrated acid, 15 cc. of 10 per cent alkali, and without adding the standard ammonium sulfate solution, this was made up to the 100 cc. mark, shaken, and filtered as above. To 50 cc. of this filtrate were added 10 cc. of the standard ammonium sulfate solution, Nesslerized, made up to 100 cc., shaken, and compared on the colorimeter, with a solution of flask A. The color intensity of these two solutions was found to be identical. The free ammonia present in flask A was, apparently, completely held in solution. This experiment was repeated three times with the same results.

As a result of these findings the following modification of the direct Nesslerization method has been adopted for the determination of total and non-protein nitrogen in spinal fluids. The same principle is, of course, available in the other applications of the micro method.

*Total Nitrogen.*—2 cc. of spinal fluid are pipetted into a test-tube, 1 cc. of the phosphoric-sulfuric acid mixture added, and the digestion carried out over a micro burner until the appearance of sulfuric acid fumes. The mouth of the test-tube is then covered with a watch-glass and heating continued for about a minute. The color obtained usually is straw-yellow. After permitting the test-tube to cool, the contents are rinsed quantitatively into a 100 cc. volumetric flask, using about 60 cc. of

water in the process. 15 cc. of 10 per cent sodium hydroxide solution are added to neutralize the cc. of concentrated acid and permit a surplus of 2 cc. of alkali. It is then made up to volume, shaken, and filtered.

Into another 100 cc. volumetric flask are pipetted 1 cc. of the concentrated acid mixture, diluted, 20 cc. of the standard ammonium sulfate solution, and an amount of 10 per cent alkali solution equal to that added to the unknown. This also is made up to volume, shaken, and filtered.

Aliquot portions of these filtrates are employed for Nesslerization, the amounts used depending on the quantity of nitrogen present in the total amount; also perhaps on the type of glassware used. Thus, if the total amount of nitrogen in the digestive mixture is equivalent to about 1 mg., the Nesslerization may be completed in several ways.

25 cc. of the water-clear filtrates of both the unknown and standard may be pipetted into two 100 cc. volumetric flasks, diluted to about 75 cc. with water, 10 cc. of Nessler solution added to each, made up to volume, shaken, and the colors compared on the colorimeter. The standard in this case will contain 0.25 mg. of nitrogen and the calculations are made accordingly. If desired, 50 cc. quantities of the filtrates may be pipetted into two 100 cc. volumetric flasks, 10 cc. of Nessler solution added to each, made up to volume, and the colors compared. In this case the standard will contain 0.5 mg. of nitrogen. Then again, 25 cc. quantities may be pipetted into 50 cc. volumetric flasks, Nesslerized, and the colors compared as above. The possibilities for error are reduced to a minimum in each case, because the standard and unknown, with the exception of the destructive digestion, go through the same preparation and filtration, side by side. Furthermore, no determination need be discarded due to an error or accident during Nesslerization. There is always a sufficient quantity of water-clear filtrate on hand to repeat the process.

The importance of obtaining water-clear filtrates cannot be overemphasized. The precipitate of calcium formed on the addition of the 10 per cent alkali is gelatinous in character (probably tertiary calcium phosphate,  $\text{Ca}_3(\text{PO}_4)_2$ ), and traces often pass through the filter paper on the first filtration. It is some-

times necessary, therefore, to pour the filtrate back into the same funnel and refilter.

*Non-Protein Nitrogen.*—For the precipitation of the proteins from the blood, Folin and Denis<sup>3</sup> employ a freshly prepared 25 per cent solution of glacial phosphoric acid. To 5 cc. of blood in a 50 cc. volumetric flask they add about 20 cc. of water and 3 cc. of glacial phosphoric acid, shake vigorously for 5 minutes or more, make up to volume, and filter through a dry filter paper. For a nitrogen determination they employ 10 cc. of the filtrate.

In view of the fact that the spinal fluid used contains relatively minute amounts of protein, it seemed unnecessary to dilute this fluid for precipitation. To 5 cc. of spinal fluid in a large sized test-tube are added quantitatively 2 cc. of a freshly prepared 25 per cent glacial phosphoric acid solution. The test-tube is then stoppered and either vigorously shaken for about 5 minutes or permitted to stand 1 to 24 hours, and filtered through a small dry filter paper. For duplicate nitrogen determinations, 3 cc. quantities of the water-clear filtrates are used for digestions. From this point the procedure employed is identical with that described for total nitrogen. In each case Nessler solution was added to water-clear filtrates and not a single determination had to be discarded.

*Urea Nitrogen.*—The direct Nesslerization method for urea determinations in the blood as outlined by Folin and Denis<sup>4</sup> was adopted for the determinations of urea in the spinal fluid, as follows:

To 5 cc. of spinal fluid in a 100 cc. volumetric flask are added about 5 cc. of water and 0.1 gm. of dry urease, shaken, and permitted to stand at room temperature from 15 to 20 minutes. This is then diluted with about 50 cc. of water, 2 cc. of freshly prepared glacial phosphoric acid added, also 0.5 gm. of Merck's blood charcoal, and made up to volume. This is shaken from time to time and allowed to stand for 45 minutes or more, when it is ready to be filtered. Definite portions of the water-clear filtrates are used for Nesslerization as in the cases of the total and non-protein nitrogen determinations.

<sup>3</sup> Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 491.

<sup>4</sup> Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 505.

In view of the fact that the aeration method for micro nitrogen determinations is still largely employed, it may not be amiss, in this connection, to point out some of the more important difficulties encountered by this procedure.

The suction created by a water pump was utilized in this laboratory, for the aspiration of ammonia into acid solutions. The velocity of the water was irregular, depending apparently at any given time on the quantity of water used in other parts of the building. Aerations, therefore, had to be carried on for comparatively long intervals—about 3 hours at a time. Even then, however, much of the work had to be discarded, because on further aspiration into fresh absorption solutions, traces of ammonia would again and again be found to have escaped aeration.

It is generally stated in text-books that 15 minutes of rapid aeration are sufficient to aspirate the ammonia completely into acid solutions. Whether this short period is sufficient is probably questionable. On many occasions in this laboratory (on holidays, for instance, when practically no one else in the building employed water), the rapidity of the air current was nearly as great as the apparatus would permit, and after 40 minutes of such aeration, traces of unaspirated ammonia were demonstrated several times.

Another factor not sufficiently emphasized in text-books is that during a rapid aeration, when the ammonia is aspirated into a single acid tube, traces of ammonia will escape absorption.

From these findings, it appears that in agreement with the statements of various other investigators,<sup>5</sup> in order to insure the complete removal of ammonia and avoid any possible loss of the same: (1) a rapid air current is absolutely essential; (2) the aeration should be carried on for about an hour; (3) the ammonia should be aspirated into two acid solutions instead of one, and these should be combined quantitatively before Nesslerization.

For a time, clouding on the addition of dilute Nessler solution presented some difficulty. This was overcome by diluting the Nessler solution further. When employing a standard of 0.5 or 0.25 mg. of nitrogen, it was found advisable to dilute the Nessler solution 1:8 instead of 1:5. If used immediately, this dilution did not give rise to the precipitation of mercury. During the hot weather, it was found advantageous also to cool the solutions under cold tap water before Nesslerization. This factor was found to minimize the possibilities of clouding on the addition of Nessler solution.

A series of parallel nitrogen determinations were carried out by means of the aeration and direct Nesslerization methods. In the case of total and non-protein nitrogen, the latter method,

<sup>5</sup> Compare, for example, Davisson, B. S., Allen, E. R., and Strubbe-field, B. M., *J. Ind. and Eng. Chem.*, 1916, viii, 896.

as modified in this laboratory, was employed. In the case of urea nitrogen, the method was used precisely as outlined by Folin and Denis.

The following is a tabulation of the results obtained.

TABLE.  
*Nitrogen per 100 Cc. of Spinal Fluid.*

No.	Direct Nesslerization method.	Aeration method.
<i>Total Nitrogen.</i>		
	<i>mg.</i>	<i>mg.</i>
1	31.25	31.25
2	17.75	17.69
3	18.55	18.30
4	17.50	17.47
5	37.72	37.35
6	21.90	21.90
7	22.52	22.72
<i>Non-Protein Nitrogen.</i>		
8	14.00	13.77
9	31.09	30.97
10	14.00	13.45
11	14.35	14.05
12	15.86	15.90
13	24.72	24.85
14	33.63	33.32
<i>Urea Nitrogen.</i>		
15	11.55	11.22
16	15.75	15.62
17	11.36	11.36
18	20.75	20.75
19	21.62	21.62
20	6.31	6.25



# THE EFFECTS OF FEEDING THE PROTEINS OF THE WHEAT KERNEL AT DIFFERENT PLANES OF INTAKE.\*

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Feeding experiments have now fully established what chemical investigations during the past 15 years have all supported; *viz.*, that there are great variations in the biological values of proteins from various sources, which depend upon the proportions of the amino-acids they yield on digestion.<sup>1</sup> It is not yet apparent whether an animal is as well off physiologically with a ration, otherwise satisfactorily constituted, but containing a high content of protein of low value as with the same food mixture with its low grade protein replaced by its biologically equivalent amount of a much better protein. In the present communication we attempt to throw light on this problem. Incidentally we have in the experiments here reported gained an insight into the tolerance of the rat for rations containing extremely high protein contents.

One of us has reported growth approximating the normal rate and successful reproduction in rats fed exclusively on boiled egg yolk, a ration which would contain not far from 31 per cent of N  $\times$  6.25.<sup>2</sup> Watson<sup>3</sup> restricted the ration of young rats to raw meat and observed high mortality, stunted growth, and pathological conditions of the thyroids, parathyroids, and kidneys.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323. McCollum, E. V., and Davis, M., *ibid.*, 1915, xx, 415. Osborne, T. B., and Mendel, L. B., *ibid.*, 1915, xx, 351.

<sup>2</sup> McCollum, *Am. J. Physiol.*, 1909-10, xxv, 120.

<sup>3</sup> Watson, C., and Hunter, A., *J. Physiol.*, 1906, xxxiv, 111.



These results afford no evidence as to the effects of a high protein diet on the well-being of an animal until it is shown that the various other factors which operate in making up a satisfactory diet are so adjusted as to lead to adequate nutrition with lower planes of protein intake. Later studies have convinced us that while egg yolk contains all the organic complexes essential for nutrition, egg yolk as the sole food does not form a very satisfactory diet when gauged by the amount of reproduction secured or by the longevity of the animals so fed. A meat diet, as employed by Watson, can be tolerated for a time, but not without detriment. We have shown in discussing the vegetarian diet<sup>4</sup> that besides the energy factor, four other factors, *viz.*, quality and amount of protein, character of the inorganic moiety, and content of the unidentified dietary factors, fat-soluble A and water-soluble B,<sup>5</sup> operate to determine whether nutrition will be satisfactory. Any one of these, if of an inadequate character, can lead to nutritive disaster.

The results of the present study may be briefly summarized as follows:

1. In agreement with our former experience with the feeding of diets high in their wheat content<sup>6</sup> we again have to emphasize the marked injury to the progeny which results from such restricted diets.

2. We have not been able to make up a ration containing wheat proteins only which was adequate for rearing of the young, although we have varied the protein content from 6.5 per cent (Chart 1) to 47.98 per cent (Chart 10). Over a wide range of protein content growth approximated the normal, but pronounced injurious effects of the ration were revealed in the reproduction records only.

3. The addition of 10 per cent of casein to a ration which contained 36.33 per cent of protein from wheat, and which was satis-

<sup>4</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333.

<sup>5</sup> McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

<sup>6</sup> Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station, Research Bull.* 17, 1911. Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.

factory with respect to all dietary factors other than protein and an inherent toxicity, improved the ration in a marked degree (Chart 12).

4. Growth was not interfered with by the inclusion of as much as 40.45 per cent of wheat proteins in the diet, but on this the young could not be reared (Chart 9).

5. Growth was normal and the production of young was good on a diet containing 46.63 per cent of protein, of which 43.0 per cent was casein, and 3.63 per cent of wheat proteins. The cause of the failure to rear the young on this diet has not yet been definitely ascertained, but would appear to be due in great part at least to the shortage of the supply of the dietary factor B, the sole source of which was the 33 per cent of wheat in the food mixture.

6. As small an amount as 15 per cent of whole wheat as the source of the water-soluble B, suffices for the completion of growth in the rat and so promotes well-being as to induce the production of a nearly normal number of young. The amount of this substance is not great enough to enable the young to develop to weaning age without causing pronounced nervous disturbances which end in death (Charts 14, 16, and 17).

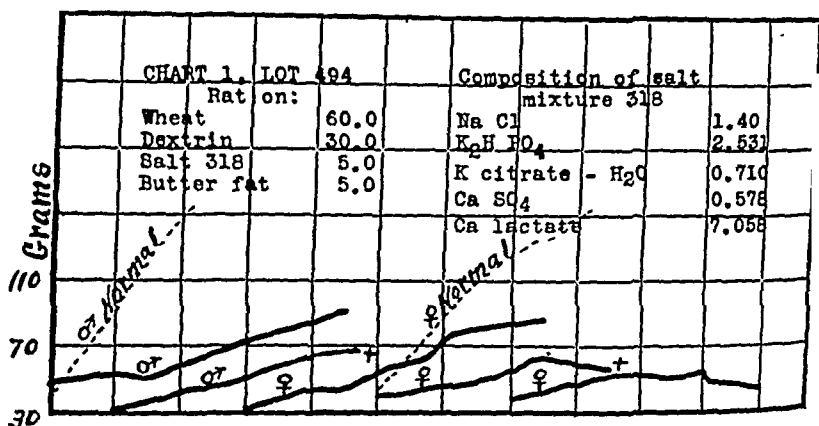


CHART 1. Lot 494 received a ration so constituted as to be entirely satisfactory except for its low content of protein of relatively poor quality. The ration contained 6.5 per cent of protein, all derived from the wheat kernel. On this plane very slow but continuous increase in body weight took place during 4½ months, when the experiment was discontinued.

The wheat furnished an abundance of the unidentified dietary factor, the water-soluble B (Chart 17); the butter fat furnished additional fat-soluble A, over the small amount carried by the wheat kernel. The salt mixture added was of a character which supplemented the inorganic content of the wheat so as to induce growth and well-being. This ration was vastly improved by the inclusion of more protein (Charts 2, 6, and 8). Y marks the birth of young.

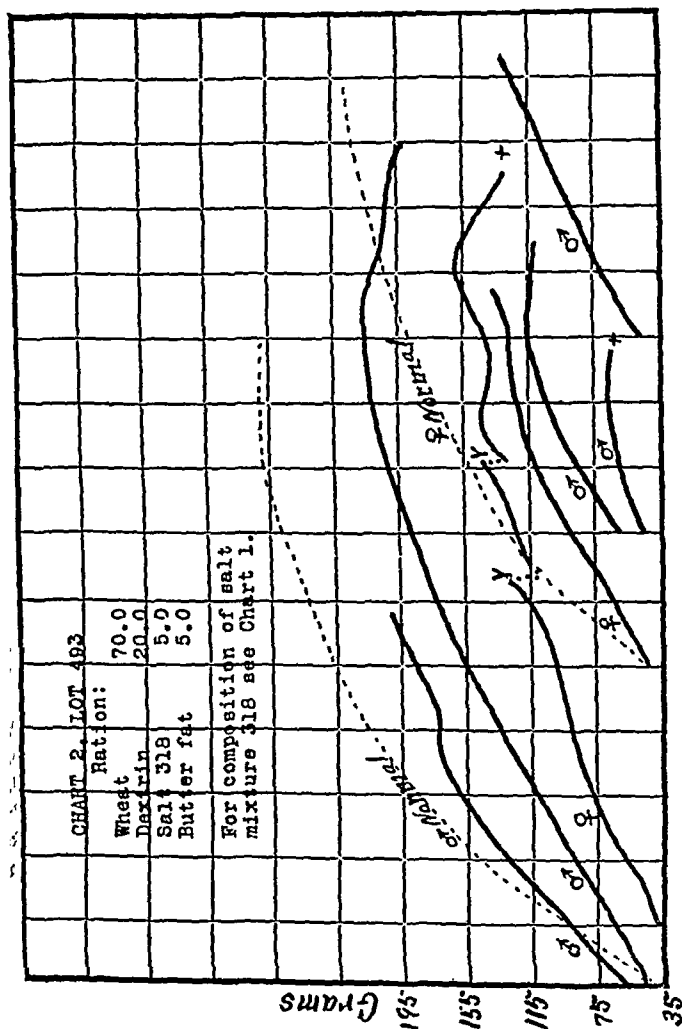


CHART 2. Lot 493 illustrates the great difference in the rate of growth of rats receiving 7.7 per cent of protein as compared with those receiving but 6.5 per cent (Chart 1). The normal adult size is never attained, however, and the span of life is short. Rats on this diet show marked signs of age at 10 to 12 months, and die at about that age. The span of life for the rat when fed a mixed diet is about 36 months. With the exception of the amount and character of the protein this food mixture is entirely adequate for complete growth and well-being (Charts 1, 6, and 8).

The two litters of young produced by one of the females in this group were not kept alive by the mother beyond the 2nd day.

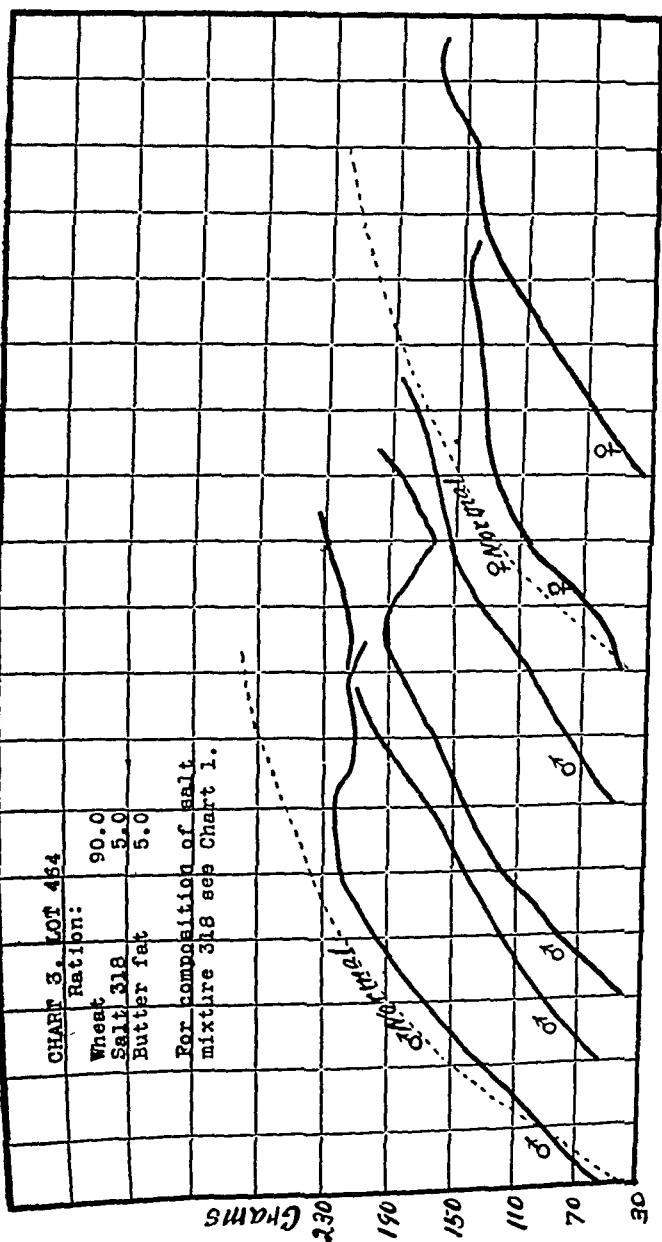


CHART 3. Lot 464 shows growth of rats at somewhat less than the normal rate, and failure to attain the full adult size on a ration otherwise satisfactory but containing only 9.9 per cent of protein, all of which was supplied by whole wheat. This amount of protein derived from the wheat *germ* is adequate for growth at the maximum possible rate.<sup>7</sup> The addition of 2.2 per cent of protein over that of Lot 493 (Chart 2) did not improve the appearance or size to the degree which one might expect.

<sup>7</sup> McCollum, Simmonds, and Pitz, *J. Biol. Chem.* 1914



of sodium salts but not always equally well with other salts; but we can demonstrate the opposite effect, namely, the antagonistic salt action, with almost any salt if the concentration is high enough. As an example we will compare the behavior of eggs in M/8 KCl made up in various concentrations of the citrates of Li, Na, and  $\text{NH}_4$ . The eggs were washed for 24 hours in  $\text{H}_2\text{O}$  before being put into the M/8 KCl solutions. Table III gives the percentage of eggs with heart beats in the three series of solutions after 5 days.

TABLE III.

	Percentage of eggs with heart beat after 5 days in M/8 KCl made up in							
	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096
$(\text{NH}_4)_3$ citrate.....	34	78	80	80	60	55	35	15
$\text{Li}_3$ citrate.....	40	50	60	33	25	10	16	33
$\text{Na}_3$ citrate.....	30	90	75	50	10	50	55	
								$\text{H}_2\text{O}$
								16
								6
								35

In all three solutions the antagonistic effect of the citrate is noticeable in concentrations of M/256 to M/64, since in all these solutions the diffusion of M/8 KCl into the egg (measured by the cessation of the heart beat) was much slower than in the pure M/8 KCl solution (made up in  $\text{H}_2\text{O}$ ). The accelerating salt effect was marked in  $\text{Na}_3$  citrate (M/512), was noticeable also in  $\text{Li}_3$  citrate (M/1024), but was lacking in  $(\text{NH}_4)_3$  citrate. As an indication of the completeness of the antagonistic effect we may state that in M/8 KCl in M/64  $\text{Na}_3$  citrate 50 per cent of the hearts were still beating after 10 days, when the experiment was discontinued, while with less  $\text{Na}_3$  citrate all the hearts stopped beating in a much shorter time.

Observations on the influence of some other salts upon the diffusion of KCl into the egg are given in Table IV. M/8 KCl was made up in solutions of these different salts as well as in M/8 sea water and in  $\text{H}_2\text{O}$ . The two latter served as controls. It is obvious how much more rapidly the eggs were poisoned when the M/8 KCl was made up in sea water than in a pure KCl solution. The concentrations in which the general salt effect is noticeable are printed in bold-faced type again. They are

not as pronounced in the case of Mg, Ca, and Sr, but it is perhaps of interest that this effect appears in a much lower concentration ( $M/1024$  or  $M/2048$ ) than in the case of NaCl, where it was  $M/8$  or  $M/4$ .

TABLE IV.

		Percentage of hearts beating in $m/8$ KCl made up in														
After		$m/8102$	$m/4096$	$m/2048$	$m/1024$	$m/512$	$m/256$	$m/128$	$m/64$	$m/32$	$m/16$	$m/8$	$m/4$	$m/2$	$m/8$ sea water.	$H_2O$
days																
4	$MgCl_2$ .....		64	40	19	39	60	65	40	30	5				0	45
5	$CaCl_2$ .....	50	40	23	50	55	50	50	60	60	25	0	0		0	25
5	$SrCl_2$ .....	40	50	10	50	60	60	55	35	45	10	0	0		0	25
4	$CsCl_2$ .....					55	23	39	41	60	42	9	9		0	10
3	$BaCl_2$ .....							85	66	65	60	35	0		5	45
4	$NH_4NO_3$ .....							70	65	65	56	58	12	0	25	15

## SUMMARY.

The addition of a second salt to an  $M/8$  KCl solution has the four following effects according to the concentration:

1. Beginning with the lowest concentration the addition of a second salt has often a slightly retarding effect upon the diffusion of KCl through the membrane into the egg.

2. With further increase of concentration of the second salt ( $M/4$  NaCl,  $M/512$  Na<sub>3</sub> citrate), an acceleration of the diffusion is noticeable (general salt effect).

3. With still further increase of the concentration ( $M/2$  to  $M/1$  NaCl,  $M/256$  to  $M/128$  Na<sub>3</sub> citrate) the opposite is noticeable, a retardation of the diffusion (antagonistic salt action). The ratio of the antagonistic to the accelerating concentration was found to be roughly about 2 : 1 for different sodium salts.

4. When the concentration is still higher the eggs are killed more rapidly again, this time probably not through the diffusion of the KCl, but of the second salt into the egg; since if we balance the second salt (e.g., NaNO<sub>3</sub> or NaCl) by the addition of a third salt (e.g., CaCl<sub>2</sub> or MnCl<sub>2</sub>) the concentration of NaCl or NaNO<sub>3</sub> required for this effect is raised.



In these experiments in which a second salt was always present with the KCl in the outside solution the antagonistic action could be more generally demonstrated than the accelerating effect. This is the reverse of what was found in the recovery experiments where only one salt was present in the outside solution and where the general salt action was clear but where no antagonistic action to KCl could be demonstrated.

# ELIMINATION OF MALATES AFTER SUBCUTANEOUS INJECTION OF SODIUM MALATE.\*†

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(Received for publication, October 22, 1916.)

Ohta<sup>1</sup> appears to be the only investigator who has studied the fate of malic acid in the animal body. He succeeded in isolating an appreciable quantity of malic acid from the urine of a dog which had received subcutaneous injections of sodium malate in large amounts. The acid thus isolated was fully identified by its melting point, its optical rotation, and by means of an elementary analysis. Ohta also described in some detail experiments on a few rabbits and dogs which had received fairly large quantities of sodium malate *per os* and by subcutaneous injection. By determining the percentage of malic acid in the urine of his experimental animals, Ohta concluded that in some cases malates escaped complete oxidation in the body. The percentage elimination was, however, always small, never exceeding 5 per cent of the amount given by mouth, and never more than 6 per cent of the amount injected subcutaneously.

The present paper gives the results of a comparative study of the elimination of malates following subcutaneous injection of sodium malate in rabbits and in cats. In view of the incompleteness of Ohta's protocols and the meagerness of his analytical data, some of his experiments have been repeated.

\* Published with the permission of the Secretary of Agriculture.

† This problem was suggested by Dr. William Salant, Pharmacologist in charge of the Pharmacological Laboratory, Bureau of Chemistry, United States Department of Agriculture.

<sup>1</sup> Ohta, K., *Biochem. Z.*, 1912, xliv, 482.

## EXPERIMENTAL.

*Analytical Procedure.*

Repetition of Ohta's work led to an examination of his method for the determination of malic acid in urine. His procedure involves a modification of Yoder's polariscopic method<sup>2</sup> for the determination of malic acid in maple products. This method depends on the enhancement of the levorotation of malic acid solutions caused by the addition of uranyl acetate.

In his preliminary experiments, which need not be given in detail, Ohta used 5 cc. of a saturated aqueous solution of uranyl acetate for every 10 cc. of normal<sup>3</sup> urine to which known quantities of malic acid had been added. He assumed that the uranyl acetate was then present *in excess*, and after filtering his mixture, polarized the clear yellow filtrate and calculated the amount of malic acid by the use of Yoder's empirical formula. The fact that he could recover 93 per cent of the malic acid added seemed to show that his technique was correct, and the procedure was followed in determining the amount of malic acid eliminated by his experimental animals.

In a series of experiments in which Ohta's method was used on normal urines containing known amounts of malic acid, the author was in some cases able to recover 88 per cent of the malic acid present. In other urines, however, the filtrate after uranyl acetate addition was optically inactive, and it was impossible to show even the presence of malates. It soon became evident that the success in following Ohta's procedure depended on the nature of the individual urines examined. In some cases phosphates or other urinary constituents caused complete precipitation of the uranium reagent, leaving an optically inactive filtrate, and thus invalidated the test. It was found necessary, therefore, to devise a modification which would insure a true excess of uranyl acetate in the filtrate used for polarization.

The following analytical procedure, involving a slight but essential modification of Ohta's method, was found satisfactory for the determination of small amounts of malate in urine. 10 cc. of urine,<sup>4</sup> neutralized (litmus) with normal NaOH or with dilute acetic acid, depending on the initial reaction to litmus, were treated with 2 cc. of a 30 per cent acetic acid solution and

<sup>2</sup> Yoder, P. A., *J. Ind. and Eng. Chem.*, 1911, iii, 569.

<sup>3</sup> Malate-free urine, which after neutralization with NaOH was acidified with varying amounts of acetic acid.

<sup>4</sup> If the urine is alkaline and contains a sediment, it is best first to render the mixture acid with hydrochloric acid, filter, and exactly neutralize the filtrate with NaOH.

diluted with water to 25 cc. (or to some other definite convenient volume). Powdered uranyl acetate was added in large excess, the mixture boiled for about 2 minutes, and vigorously shaken. After heating, solid uranyl acetate still remained in contact with the solution. Upon cooling to room temperature, the solution was again diluted to 25 cc. (or to the volume which obtained prior to heating), and the mixture allowed to stand no less than 5 hours in a dark cupboard. At the end of this period the solution was filtered into a 200 mm. or a 100 mm. observation tube and polarized at about 20°, using white light and the bichromate cell. The Bates saccharimeter was used in all determinations, the scale reading in degrees Ventzke. The percentage of malic acid present in the solution was found from data tabulated by Gore.<sup>5</sup> By a simple proportion the amount of malic acid in the initial volume of solution was computed and the weight of sodium malate estimated.

Table I shows with what success the above method may be applied and also indicates the limitations of the method.

TABLE I.

*Results of Preliminary Experiments on a Method for the Determination of Malic Acid in Urine.*

Urine (normal).	Malic acid added to 10 cc. of urine.	Malic acid recovered.
	mg.	mg.
Human.....	12.5	14.4
“.....	25	24
“.....	50	49.8
“.....	125	118
“.....	125	116
“.....	100	94
Rabbit.....	37.5	37.2
“.....	50	50.6
“.....	50	49.2
“.....	100	93.8
“.....	None.	None.
Cat.....	100	95
“.....	100	94.6

<sup>5</sup> Gore, H. C., Report on Fruit and Fruit Products, *J. Assn. Off. Agric. Chem.*, 1915, i, 481.

Experiments have shown that the above procedure may be carried out with slight modification in the presence of glucose. In glycosuria, it is essential to make a polarimetric reading<sup>6</sup> prior to the addition of uranyl acetate, and to make allowance for the dextrorotation by adding it to the levo reading obtained after addition of the uranium reagent.

### *Animal Experiments.*

Aqueous solutions used for injection ordinarily contained 100 gm. of anhydrous sodium malate per liter of solution, and are referred to in the protocols as 10 per cent solutions. When occasional large injections were necessary, 25 per cent solutions were used. Urines of the experimental animals were preserved with toluene and were analyzed as soon as possible after collection. In each experiment a convenient aliquot portion of the total urine voided during a 24 or 48 hour pre-observational period was polariscopically examined according to the above procedure. In a few cases such urines showed a negligible optical activity; ordinarily they were optically inactive. Aliquot portions of the urine eliminated during the 22 to 24 hour period following injection were examined and their malate content was determined. The urine of a second 24 hour period was also analyzed. In most cases all of the undestroyed malic acid (found in the urine) was eliminated in the course of the first 24 hours following malate injection. In some individuals, however, small but appreciable quantities of malate could be recovered from the urine the 2nd day following injection. In such cases the amount eliminated was so small that further search for malate was ordinarily deemed unnecessary.

### *Experiments with Oat-Fed Rabbits.*

Injection of sodium malate was in all cases followed by marked elimination of malates in the urine. In two cases, 3.3 gm. of sodium malate per kilo of body weight were injected, but as one of our experimental animals (Rabbit 1) died within 48 hours after such an injection, the dose was decreased to 1 gm. per kilo in our other experiments. The protocol of Rabbit 1 follows. It should be noted that the percentage elimination of malate is in

<sup>6</sup>The error in glucose reading, owing to the very slight levorotation of the malic acid, is negligible.

## PROTOCOL I.

*Subcutaneous Injection of Oat-Fed Rabbit 1 with 5.8 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Urine eliminated.	Data relating to malate elimination in urine.
Oct. 30 Nov. 1	9.00 a.m.	Weight 1.705 kg. 48 hr. urine collected.	80 cc.	Urine was optically inactive after uranyl acetate addition. <i>Malates and other optically active substances absent.</i>
	11.20 "	Injected subcutaneously (two sites) 22.5 cc. of a 25 per cent sodium malate solution (i.e., 5.63 gm. of sodium malate).		
	11.50 "	No symptoms noted.		
	1.30 p.m.	No symptoms other than diarrhea.		
	4.30 "	No further symptoms noted.		
" 2	9.00 a.m.	Muscular incoordination and tremors noted. Marked weakness of the extremities.		Dextrorotatory prior to uranyl acetate addition. Total elimination corresponds to 970 mg. of sodium malate (due correction being made for glucose) = 17 per cent of the total malate injected.
	9.30 "	24.5 hr. urine collected.	100 cc. Urine alkaline. <i>Glycosuria.</i>	
" 3	9.00 "	Rabbit found dead.		

TABLE III.  
*Summary of Experiments on Carrot-Fed Rabbits.*

Rabbit.	Sex.	Description.	Weight.	Sodium malate injected per kg. of body weight.	Sodium malate injected.	Sodium malate eliminated.	
						gm.	per cent
8	♀	Maltese.	2.075	1.0	2.08	0.24	11.5
9	♀	"	2.33	1.0	2.35	0.38	16.0
10	♂	White.	1.445	1.0	1.45	0.26	18.0
11	♂	Belgian.	2.335	1.0	2.35	0.25	10.5
Average ..			.....	.....	.....	.....	14.0

observation in separate cages for a month or more. The cats were in good condition and ate normally at the outset of the present experiments.

A preliminary trial, in which doses of 3.3 gm. of sodium malate per kilo of body weight were subcutaneously injected into two cats, resulted in the death of one animal, whose urine during the observational period was not examined. In consequence the dose was decreased to 1 gm. per kilo. In general, these smaller doses caused no symptoms or, at most, a slight general depression. Malates were invariably found in the urine after injection, as shown in Table IV.

TABLE IV.  
*Summary of Experiments on Cats.*

Cat.	Sex.	Description.	Weight.	Dose per kg.	Sodium malate injected.	Sodium malate eliminated.
			kg.			
1	♀	White and tiger.	2.4	1.0		
2	♂	Black and white.	2.4			
3	♀	Black.	2.385			
4	♂	Gray and white.	1.83			
5	♀	White and ti	2.45			
6	♂	" "	1.98			
6*	♂		12			
Average. ....			.....	.....	.....	.....

\* Reinjected injection.

# PROTOCOL

*Subcutaneous Injection of Cat 2\* with 1 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Rations.		Urine voided.	Data relating to malate elimination in urine.
			Meat.	Water.		
			gm.	cc.		
Nov. 15	9.00 a.m.	Condition of animal good. 48 hr. urine collected.	100	40	76 cc. Faint trace of albumin. No glycosuria.	Urine inactive after addition of uranyl acetate. <i>Malates absent.</i>
	10.50 "	Injected subcutaneously 24 cc. of a 10 per cent sodium malate solution.				
	1.05 p.m.	No symptoms noted.				
	2.50 "	No symptoms noted.				
	4.25 "	No symptoms noted.				
" 16	9.00 a.m.	Animal apparently normal.	100	80	56 cc. Free from albumin and sugar.	Test for malates made on 10 cc. of urine showed rotation of $-3.00^{\circ}$ V. in a 100 mm. tube. This corresponds to a total elimination of 410 mg. of sodium malate; i.e., a 17 per cent elimination.
" 17	9.00 a.m.		100	30	56 cc.	Test on 10 cc. of urine showed rotation of $-0.35^{\circ}$ V. Total elimination of 50 mg. of sodium malate; i.e., 2 per cent.
" 18	9.00 "		100	20	58 cc.	<i>Malates absent.</i>

\* Received 1 gm. of sodium citrate per kg. subcutaneously, on Oct. 4, 1915. Weight of animal on Nov. 13, 1915, 2.4 kg.



PROTOCOL IV.

*Subcutaneous Injection of Cat 1\* with 1 Gm. of Sodium Malate per Kg.*

Date.	Time.	Remarks.	Rations.		Urine voided.	Data relating to malate elimination in urine.
			Meat.	Water.		
			gm.	cc.		
Nov. 13	9.00 a.m.	Weight 2.4 kg. Condition good.				
" 15	9.00 "	48 hr. urine collected (49 hr. rations).	100	60	102 cc. containing neither albumin nor sugar.	Test for malates on 10 cc. of urine negative.
	10.55 "	Injected subcutaneously 24 cc. of a 10 per cent sodium malate solution.				
	1.05 p.m.	No symptoms noted.				
	2.50 "	No symptoms noted.				
	4.25 "	No symptoms noted.				
" 16	9.00 a.m.	Animal apparently normal.	100	90	105 cc. No albumin and no sugar.	Test on 10 cc. of urine shows a rotation of $-3.55^{\circ}$ V. in 100 mm. tube. This reading corresponds to a total elimination of 900 mg. of sodium malate; i.e., 37.5 per cent elimination.
" 17	9.00 "	Animal apparently normal.	100	20	85 cc.	Test for malates on 10 cc. of urine negative.

\* Received 1 gm. of sodium citrate per kg. subcutaneously, on Oct. 7, 1915.

## DISCUSSION.

As shown by the results of preliminary experiments, the quantity of uranyl acetate necessary to permit the quantitative estimation of malates in the urine of certain individual animals is much greater than the amounts used by Ohta in his work. It appeared possible therefore that Ohta himself did not in every case secure a quantitative determination of the malates present in the urine. As a matter of fact, the analytical figures given above indicate strongly that his data were subject to error. His general conclusion that *malates are incompletely destroyed in the body* is substantiated by the figures given above, and indeed more strikingly than by his own. Whereas Ohta was never able to recover from the urine more than 6 per cent of the amount of malate injected into rabbits, and was in the case of one urine unable to demonstrate the presence of malates, the results of the present paper show an elimination of from 3 to 21 per cent of the total malate injected. Comparative data obtained with oat-fed rabbits on the one hand, and carrot-fed rabbits on the other, indicate that the effects of diet on malate elimination are negligible in the case of these animals. Without wishing to overemphasize an average of results which themselves cover a rather wide range, it may be well to point out that rabbits eliminate, on the average, 14 per cent of the malate injected, whereas the average elimination in cats is 26 per cent.

The lowest elimination in the case of rabbits was 3 per cent; the highest was roughly 21 per cent. The lowest malate elimination in the case of cats was about 17 per cent, the highest exceeded 41 per cent of the amount injected. A real difference therefore appears to exist between cats and rabbits in their ability to destroy malates after subcutaneous injection.

## SUMMARY AND CONCLUSIONS.

1. The estimation of small amounts of malic acid in urine was satisfactorily accomplished by using a modified Ohta-Yoder method. The essential modification consisted in *saturating* the urine with *powdered* uranyl acetate, instead of adding a *saturated solution* of the reagent.

2. Sodium malate, injected subcutaneously in moderate doses,

was shown to be incompletely destroyed by rabbits and cats, and was eliminated in the urine. Rabbits eliminated from 3 to 21 per cent, whereas cats eliminated from 17 to 41.5 per cent of the total amount of malate injected.

3. Elimination of undestroyed malate in the urine of the experimental animals was usually complete within 24 hours after injection.

4. Subcutaneous injections of sodium malate in amounts *not exceeding* 1 gm. per kilo were *not* followed by nephritis or glycosuria.

5. Rabbits showed no toxic symptoms after receiving subcutaneous injections of sodium malate in amounts *not exceeding* 1 gm. per kilo. Subcutaneous injections of corresponding amounts of sodium malate into cats caused either no symptoms or at most a slight temporary depression.

6. Toxic symptoms in both cats and rabbits followed the injection of larger amounts (3.3 gm. per kilo) of sodium malate. No effort, however, has been made to fix the lowest limit of the toxicity of sodium malate.

# THE RELATION BETWEEN THE WATER AND GLUCOSE CONCENTRATION OF THE BLOOD.

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Polyuria, edema, and desiccation are all characteristic manifestations of diabetes mellitus. These changes in the water metabolism are generally regarded as the result of glycosuria and the increase of glucose in the fluids and tissues of the body. All attempts to associate hydremic or inspissated blood with a parallel increase or diminution of the blood sugar have thus far failed, and Morawitz,<sup>1</sup> in summarizing the situation, shows how the blood of the diabetic has been found by some investigators to be inspissated, while others have demonstrated a hydremia. From the therapeutic point of view, these questions are of great importance, for on their solution depends the regulation of the diabetic's water intake. Applying micro methods to this problem, it was possible to obtain the blood in 10 to 15 minute intervals, determine the sugar and water content in each specimen, and note the relation between the two. Where it was desired to raise the sugar content of the blood, glucose was given by mouth.

The method of Lewis and Benedict<sup>2</sup> was used to determine the blood sugar. The water content of the blood was ascertained by putting about 1 cc. of freshly drawn blood into a tin capsule of known weight, weighing immediately to determine the quantity of blood, placing the capsule, after removal of the cover, in a drying oven at 98°C. for 24 hours, then allowing it to remain in a desiccator for 48 hours, or until a constant weight was established; after weighing again, the percentage of water lost could be calculated. This method has errors which are apparent. However, it yields results which are comparative, the error being constant. Good duplicates are obtained with this procedure, as may be seen in Table I.

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<sup>1</sup> Morawitz, P., Oppenheimer's Handb. Biochem. Menschen u. Thiere, Jena, 1910, iv, 251. The bibliography of the subject is detailed here.

<sup>2</sup> Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

1.2 per cent, figures comparable to those obtained in the control cases (Table II). What caused the one very high figure for hydremia in H. is an open question. In G. a large volume of urine was put out constantly during the period of observation. This apparently did not affect the water concentration of the blood.

The exhibition of 100 gm. of glucose in normal individuals has no effect in changing the concentration of the blood. Polyuria observed during the course of 3 hours does not necessarily result in variations of the water content of the blood.

TABLE III.

*Data on Normal Individuals Receiving 100 Gm. of Glucose.*

Case.	Time.	Blood.		Urine volume per hr.
		Glucose.	Water.	
		per cent	per cent	cc.
H. Normal.	8.45 a.m.			
	8.47 " 100 gm. of glucose in 300 cc. of water.	0.090	76.6	
	9.00 " 15 min. interval.	0.085	78.9	7
	9.15 " 15 " "	0.124	76.7	25
	9.31 " 16 " "	0.120	77.9	64
	9.45 " 14 " "	0.099	77.7	96
	10.00 " 15 " "	0.080	77.4	72
	10.30 " 30 " "	0.072	77.6	52
	11.01 " 31 " "	0.062	77.4	69
	12.03 p.m. 62 " "	0.077	76.6	130
G. Normal.	8.35 a.m.	0.109	79.2	120
	8.42 " 100 gm. of glucose in 300 cc. of water.			
	8.55 " 20 min. interval.	0.112	78.9	215
	9.10 " 15 " "	0.132	79.3	137
	9.30 " 20 " "	0.137	79.6	320
	9.49 " 19 " "	0.111	78.6	215
	10.27 " 38 " "	0.103	78.8	160
	11.28 " 61 " "	0.109	79.1	135

*Cases of Diabetes Mellitus Receiving 100 Gm. of Glucose.*—A few of these cases are detailed in Table IV. In patients E. and C. hydremia and glycemia show no parallel whatever. In fact, the water concentration of the blood runs a course much as it does in

TABLE IV.

*Relation between Water Concentration and Glucose of the Blood in Diabetics in Whom a Hyperglycemia Is Produced by the Administration of Glucose.*

Case	Time.	Blood.		Urine volume per hr.
		Glucose	Water.	
		per cent	per cent	cc
R. Diabetes mellitus.	8 45 a.m.	0 137	78 9	76
	8 46 " 100 gm. of glucose in 300 cc. of water.			
	9 10 " 25 min. interval.	0 171	78 9	205
	9 25 " 15 " "	0 200	78 8	77
	10 07 " 42 " "	0 240	79 2	50
	11 07 " 60 " "	0 238	79 6	88
	11 36 "			83
	12 10 p.m. 63 " "	0 123	79 3	48
B. Diabetes mellitus.	8 52 a.m.	0 070	79 6	172
	8 55 " 40 gm. of glucose in 300 cc. of water.			
	9 12 " 20 min. interval.	0 090	78 8	117
	9 25 " 13 " "	0.186	79 5	134
	9 40 " 100 cc. of water.			
	9 43 " 18 min. interval.	0 210	78 0	312
	10 32 " 49 " "	0.218	77.2	380
	10 40 " 100 cc. of water.			
E. Diabetes mellitus.	11 30 " 58 min. interval.	0 210	77 8	34
	9 20 a.m.	0 108	79 7	
	9 25 " 100 gm. of glucose in 300 cc. of water.			
	9 45 " 25 min. interval.	0 126	79 3	138
	10 45 " 60 " "	0 171	78 9	
	10 58 "			895
	11 55 " 70 " "	0 173	79 6	
	12 20 p.m.			365
C. Diabetes mellitus.	12 45 " 50 " "	0.126	79 7	105
	8 45 a.m.	0 091	78 1	142
	8 48 " 100 gm. of glucose in 300 cc. of water.			
	9 10 " 25 min. interval.	0 124	77 6	122
	9 30 " 20 " "	0.210		110
	9 50 " 20 " "	0 190	78 3	83
	10 25 " 35 " "	0 174	78 6	94
	11 07 " 42 " "	0 155	78 6	102
	12 00 n. 53 " "	0 094	78 0	52

TABLE IV—Concluded.

Case.	Time.		Blood.		Urine volume per hr.  cc.
			Glucose.	Water.	
D. Diabetes mellitus.	Mar. 1	Glycemia as ob-	0.356	79.0	
	" 15	served in the	0.146	79.4	
	" 17	course of the disease. No glu- cose adminis- tered.	0.134	79.7	

the control individuals in whom no hyperglycemia was produced. How independent these two factors are of one another is seen in the last case, D., of Table IV, where a glycemia of 0.356 per cent was accompanied by a lower hydremia than a blood sugar of 0.134 per cent. In a very few cases (R., Table IV), a suggestive parallelism may be observed between the water and glucose content of the blood; in others (B., Table IV), an apparent effect of polyuria in inspissating the blood is manifested. Various authors have noted a tendency on the part of the blood and tissues to lose water<sup>5</sup> during the final stages of diabetes. It is probable that the excessive polyuria in such cases is the controlling factor.

#### CONCLUSIONS.

The water concentration of the blood in the normal individual often shows a variation of about 1 per cent during the course of a few minutes. There is no assignable cause for this inconstancy. Raising or lowering the sugar content of the blood does not give rise to a corresponding change in its concentration. If glycemia plays a part in controlling the water content of the blood, this is so slight in its effect that other factors, such as polyuria, etc., nullify its results, except in rare instances.

<sup>5</sup> Magnus-Levy, A., *Arch. exp. Path. u. Pharm.*, 1901, xlv, 389. Rumpf, T., *Z. klin. Med.*, 1902, xlv, 260. Dennstedt, M., and Rumpf, T., *Z. klin. Med.*, 1906, lviii, 84. Von Noorden, C., *Metabolism and Practical Medicine*, Chicago, 1907, iii, 614.

# THE FOLIN AND DENIS METHOD OF NITROGEN DETERMINATIONS BY DIRECT NESSLERIZATION, AND ITS APPLICATION TO SPINAL FLUIDS.

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Folin and Denis<sup>1</sup> have recently modified the micro method for nitrogen determinations, by substituting phosphoric acid for part of the sulfuric acid ordinarily employed in the destructive digestion of the nitrogenous material and by reducing the amount of free alkali in Nessler's reagent. These changes have enabled them to Nesslerize directly the ammonia produced during the digestive process, thus eliminating the aeration procedure connected with these determinations.

In attempting to apply this method to nitrogen determinations in spinal fluids, a serious difficulty has been encountered. Folin and Denis have already directed attention to the turbidity of the Nesslerized solutions due to the presence of silicates formed by the action of phosphoric acid and heat on the glass during the destructive digestion. They advise the removal of these silicates either by centrifugation or by filtration through a small cotton plug and state, "if the sediment obtained is mixed with a red deposit, the Nesslerization has not been successful and the determination must be discarded."

The difficulty just discussed has actually presented itself in this laboratory in every determination carried out according to the original directions. Furthermore, the standard solution also invariably showed clouding, indicating that the turbidity is not due to the silicates alone. In these trials, the concentrated acid mixture employed consisted of three volumes of concentrated phosphoric acid, one volume of concentrated sulfuric acid, and one-fifteenth volume of 10 per cent copper sulfate solution. The two acids were first mixed and allowed to stand over night

<sup>1</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.



to facilitate the precipitation of calcium. The mixture was then filtered by means of a Buchner funnel, through a dry mat of asbestos, after which the copper sulfate solution was added.

15 cc. of 10 per cent sodium hydroxide were found to be sufficient to neutralize 1 cc. of the concentrated acid mixture and permit a surplus of 2 cc. of alkali in the solution. This was determined in the following manner. 1 cc. of the concentrated acid mixture on titration with 10 per cent sodium hydroxide, with phenolphthalein as an indicator, required 9.5 cc. of alkali for neutralization. About three-fourths of this acidity (or 7.1 cc.) was due to phosphoric acid. As pointed out by Folin and Denis, one-third of the base combining power of the phosphoric acid is not included in the titration value. Therefore, 9.5 cc. + 3.5 cc., or 13 cc., were required for the neutralization of the acid. In order to permit a surplus of 2 cc. of 10 per cent sodium hydroxide, 15 cc. of the alkali were employed.

Nessler solution was prepared precisely as directed by Folin and Denis.

An attempt to ascertain the nature of the precipitate responsible for the turbidity in the standard disclosed by physical and chemical examination the probable presence of calcium. The precipitate of calcium formed on mixing the concentrated phosphoric and sulfuric acids, as stated above, is filtered off through a mat of asbestos. Traces of calcium, however, are soluble in an acid solution. When, therefore, the solution is rendered alkaline by the addition of 10 per cent sodium hydroxide, these traces separate out and cause turbidity on Nesslerization.

Having thus been unable to prepare clear solutions for color comparisons, an attempt was made to *filter off the precipitate before Nesslerization*. The results were entirely satisfactory. Nessler solution was thus added to water-clear filtrates in each case and clear solutions for color comparisons were obtained without exception.\*

\* It is likely that due to a higher purity of chemicals employed in the laboratory of Folin and Denis, no precipitation occurred on addition of the 10 per cent alkali. However, as is indicated by their statement quoted above, apparently some determinations have to be discarded from time to time on account of the red deposit mixed with the precipitate. With the modification suggested, no determination need ever be discarded, as is pointed out in the text later.

The probable loss of ammonia during the filtration process early suggested itself. On addition of a sufficient quantity of 10 per cent sodium hydroxide to neutralize the concentrated acid and permit a surplus of 2 cc. of alkali, ammonia is set free in the solution. Inasmuch as ammonia is extremely volatile, it was feared that traces of it might be lost during the filtration process. It was found, however, that owing to the extreme solubility of ammonia, significant losses do not occur. This was determined in the following manner.

To a 100 cc. volumetric flask, A, were added 1 cc. of the concentrated acid mixture, 15 cc. of alkali, and 20 cc. of the standard ammonium sulfate solution containing 1 mg. of nitrogen, made up to the mark, shaken, and filtered. 50 cc. of the water-clear filtrate were measured out into another 100 cc. volumetric flask, 10 cc. of Nessler solution added, made up to the mark, and shaken. To a volumetric flask, B, were added 1 cc. of concentrated acid, 15 cc. of 10 per cent alkali, and without adding the standard ammonium sulfate solution, this was made up to the 100 cc. mark, shaken, and filtered as above. To 50 cc. of this filtrate were added 10 cc. of the standard ammonium sulfate solution, Nesslerized, made up to 100 cc., shaken, and compared on the colorimeter, with a solution of flask A. The color intensity of these two solutions was found to be identical. The free ammonia present in flask A was, apparently, completely held in solution. This experiment was repeated three times with the same results.

As a result of these findings the following modification of the direct Nesslerization method has been adopted for the determination of total and non-protein nitrogen in spinal fluids. The same principle is, of course, available in the other applications of the micro method.

*Total Nitrogen.*—2 cc. of spinal fluid are pipetted into a test-tube, 1 cc. of the phosphoric-sulfuric acid mixture added, and the digestion carried out over a micro burner until the appearance of sulfuric acid fumes. The mouth of the test-tube is then covered with a watch-glass and heating continued for about a minute. The color obtained usually is straw-yellow. After permitting the test-tube to cool, the contents are rinsed quantitatively into a 100 cc. volumetric flask, using about 60 cc. of

water in the process. 15 cc. of 10 per cent sodium hydroxide solution are added to neutralize the cc. of concentrated acid and permit a surplus of 2 cc. of alkali. It is then made up to volume, shaken, and filtered.

Into another 100 cc. volumetric flask are pipetted 1 cc. of the concentrated acid mixture, diluted, 20 cc. of the standard ammonium sulfate solution, and an amount of 10 per cent alkali solution equal to that added to the unknown. This also is made up to volume, shaken, and filtered.

Aliquot portions of these filtrates are employed for Nesslerization, the amounts used depending on the quantity of nitrogen present in the total amount; also perhaps on the type of glassware used. Thus, if the total amount of nitrogen in the digestive mixture is equivalent to about 1 mg., the Nesslerization may be completed in several ways.

25 cc. of the water-clear filtrates of both the unknown and standard may be pipetted into two 100 cc. volumetric flasks, diluted to about 75 cc. with water, 10 cc. of Nessler solution added to each, made up to volume, shaken, and the colors compared on the colorimeter. The standard in this case will contain 0.25 mg. of nitrogen and the calculations are made accordingly. If desired, 50 cc. quantities of the filtrates may be pipetted into two 100 cc. volumetric flasks, 10 cc. of Nessler solution added to each, made up to volume, and the colors compared. In this case the standard will contain 0.5 mg. of nitrogen. Then again, 25 cc. quantities may be pipetted into 50 cc. volumetric flasks, Nesslerized, and the colors compared as above. The possibilities for error are reduced to a minimum in each case, because the standard and unknown, with the exception of the destructive digestion, go through the same preparation and filtration, side by side. Furthermore, no determination need be discarded due to an error or accident during Nesslerization. There is always a sufficient quantity of water-clear filtrate on hand to repeat the process.

The importance of obtaining water-clear filtrates cannot be overemphasized. The precipitate of calcium formed on the addition of the 10 per cent alkali is gelatinous in character (probably tertiary calcium phosphate,  $\text{Ca}_3(\text{PO}_4)_2$ ), and traces often pass through the filter paper on the first filtration. It is some-

times necessary, therefore, to pour the filtrate back into the same funnel and refilter.

*Non-Protein Nitrogen.*—For the precipitation of the proteins from the blood, Folin and Denis<sup>3</sup> employ a freshly prepared 25 per cent solution of glacial phosphoric acid. To 5 cc. of blood in a 50 cc. volumetric flask they add about 20 cc. of water and 3 cc. of glacial phosphoric acid, shake vigorously for 5 minutes or more, make up to volume, and filter through a dry filter paper. For a nitrogen determination they employ 10 cc. of the filtrate.

In view of the fact that the spinal fluid used contains relatively minute amounts of protein, it seemed unnecessary to dilute this fluid for precipitation. To 5 cc. of spinal fluid in a large sized test-tube are added quantitatively 2 cc. of a freshly prepared 25 per cent glacial phosphoric acid solution. The test-tube is then stoppered and either vigorously shaken for about 5 minutes or permitted to stand 1 to 24 hours, and filtered through a small dry filter paper. For duplicate nitrogen determinations, 3 cc. quantities of the water-clear filtrates are used for digestions. From this point the procedure employed is identical with that described for total nitrogen. In each case Nessler solution was added to water-clear filtrates and not a single determination had to be discarded.

*Urea Nitrogen.*—The direct Nesslerization method for urea determinations in the blood as outlined by Folin and Denis<sup>4</sup> was adopted for the determinations of urea in the spinal fluid, as follows:

To 5 cc. of spinal fluid in a 100 cc. volumetric flask are added about 5 cc. of water and 0.1 gm. of dry urease, shaken, and permitted to stand at room temperature from 15 to 20 minutes. This is then diluted with about 50 cc. of water, 2 cc. of freshly prepared glacial phosphoric acid added, also 0.5 gm. of Merck's blood charcoal, and made up to volume. This is shaken from time to time and allowed to stand for 45 minutes or more, when it is ready to be filtered. Definite portions of the water-clear filtrates are used for Nesslerization as in the cases of the total and non-protein nitrogen determinations.

<sup>3</sup> Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 491.

<sup>4</sup> Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 505.

In view of the fact that the aeration method for micro nitrogen determinations is still largely employed, it may not be amiss, in this connection, to point out some of the more important difficulties encountered by this procedure.

The suction created by a water pump was utilized in this laboratory, for the aspiration of ammonia into acid solutions. The velocity of the water was irregular, depending apparently at any given time on the quantity of water used in other parts of the building. Aerations, therefore, had to be carried on for comparatively long intervals—about 3 hours at a time. Even then, however, much of the work had to be discarded, because on further aspiration into fresh absorption solutions, traces of ammonia would again and again be found to have escaped aeration.

It is generally stated in text-books that 15 minutes of rapid aeration are sufficient to aspirate the ammonia completely into acid solutions. Whether this short period is sufficient is probably questionable. On many occasions in this laboratory (on holidays, for instance, when practically no one else in the building employed water), the rapidity of the air current was nearly as great as the apparatus would permit, and after 40 minutes of such aeration, traces of unaspirated ammonia were demonstrated several times.

Another factor not sufficiently emphasized in text-books is that during a rapid aeration, when the ammonia is aspirated into a single acid tube, traces of ammonia will escape absorption.

From these findings, it appears that in agreement with the statements of various other investigators,<sup>5</sup> in order to insure the complete removal of ammonia and avoid any possible loss of the same: (1) a rapid air current is absolutely essential; (2) the aeration should be carried on for about an hour; (3) the ammonia should be aspirated into two acid solutions instead of one, and these should be combined quantitatively before Nesslerization.

For a time, clouding on the addition of dilute Nessler solution presented some difficulty. This was overcome by diluting the Nessler solution further. When employing a standard of 0.5 or 0.25 mg. of nitrogen, it was found advisable to dilute the Nessler solution 1:8 instead of 1:5. If used immediately, this dilution did not give rise to the precipitation of mercury. During the hot weather, it was found advantageous also to cool the solutions under cold tap water before Nesslerization. This factor was found to minimize the possibilities of clouding on the addition of Nessler solution.

A series of parallel nitrogen determinations were carried out by means of the aeration and direct Nesslerization methods. In the case of total and non-protein nitrogen, the latter method,

<sup>5</sup> Compare, for example, Davisson, B. S., Allen, E. R., and Strublefield, B. M., *J. Ind. and Eng. Chem.*, 1916, viii, 896.

as modified in this laboratory, was employed. In the case of urea nitrogen, the method was used precisely as outlined by Folin and Denis.

The following is a tabulation of the results obtained.

TABLE.

*Nitrogen per 100 Cc. of Spinal Fluid.*

No.	Direct Nesslerization method.	Aeration method.
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*Total Nitrogen.*

	mg.	mg.
1	31.25	31.25
2	17.75	17.69
3	18.55	18.30
4	17.50	17.47
5	37.72	37.35
6	21.90	21.90
7	22.52	22.72

*Non-Protein Nitrogen.*

8	14.00	13.77
9	31.09	30.97
10	14.00	13.45
11	14.35	14.05
12	15.86	15.90
13	24.72	24.85
14	33.63	33.32

*Urea Nitrogen.*

15	11.55	11.22
16	15.75	15.62
17	11.36	11.36
18	20.75	20.75
19	21.62	21.62
20	6.31	6.25

These results afford no evidence as to the effects of a high protein diet on the well-being of an animal until it is shown that the various other factors which operate in making up a satisfactory diet are so adjusted as to lead to adequate nutrition with lower planes of protein intake. Later studies have convinced us that while egg yolk contains all the organic complexes essential for nutrition, egg yolk as the sole food does not form a very satisfactory diet when gauged by the amount of reproduction secured or by the longevity of the animals so fed. A meat diet, as employed by Watson, can be tolerated for a time, but not without detriment. We have shown in discussing the vegetarian diet<sup>4</sup> that besides the energy factor, four other factors, *viz.*, quality and amount of protein, character of the inorganic moiety, and content of the unidentified dietary factors, fat-soluble A and water-soluble B,<sup>5</sup> operate to determine whether nutrition will be satisfactory. Any one of these, if of an inadequate character, can lead to nutritive disaster.

The results of the present study may be briefly summarized as follows:

1. In agreement with our former experience with the feeding of diets high in their wheat content<sup>6</sup> we again have to emphasize the marked injury to the progeny which results from such restricted diets.

2. We have not been able to make up a ration containing wheat proteins only which was adequate for rearing of the young, although we have varied the protein content from 6.5 per cent (Chart 1) to 47.98 per cent (Chart 10). Over a wide range of protein content growth approximated the normal, but pronounced injurious effects of the ration were revealed in the reproduction records only.

3. The addition of 10 per cent of casein to a ration which contained 36.33 per cent of protein from wheat, and which was satis-

<sup>4</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333.

<sup>5</sup> McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

<sup>6</sup> Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station, Research Bull.* 17, 1911. Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.

factory with respect to all dietary factors other than protein and an inherent toxicity, improved the ration in a marked degree (Chart 12).

4. Growth was not interfered with by the inclusion of as much as 40.45 per cent of wheat proteins in the diet, but on this the young could not be reared (Chart 9).

5. Growth was normal and the production of young was good on a diet containing 46.63 per cent of protein, of which 43.0 per cent was casein, and 3.63 per cent of wheat proteins. The cause of the failure to rear the young on this diet has not yet been definitely ascertained, but would appear to be due in great part at least to the shortage of the supply of the dietary factor B, the sole source of which was the 33 per cent of wheat in the food mixture.

6. As small an amount as 15 per cent of whole wheat as the source of the water-soluble B, suffices for the completion of growth in the rat and so promotes well-being as to induce the production of a nearly normal number of young. The amount of this substance is not great enough to enable the young to develop to weaning age without causing pronounced nervous disturbances which end in death (Charts 14, 16, and 17).



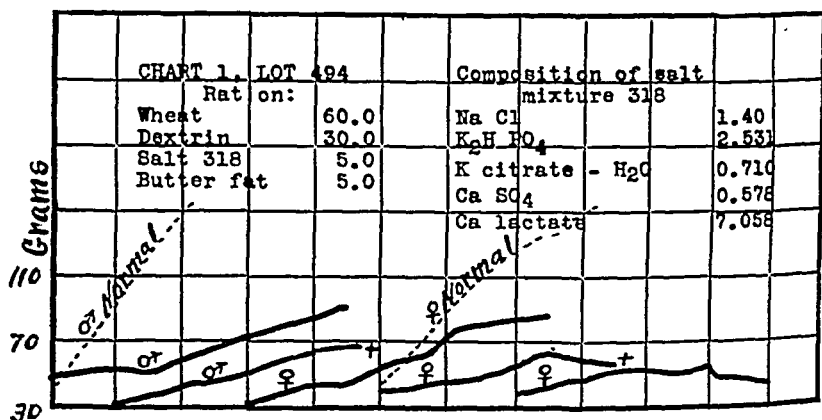


CHART 1. Lot 494 received a ration so constituted as to be entirely satisfactory except for its low content of protein of relatively poor quality. The ration contained 6.5 per cent of protein, all derived from the wheat kernel. On this plane very slow but continuous increase in body weight took place during 4½ months, when the experiment was discontinued.

The wheat furnished an abundance of the unidentified dietary factor, the water-soluble B (Chart 17); the butter fat furnished additional fat-soluble A, over the small amount carried by the wheat kernel. The salt mixture added was of a character which supplemented the inorganic content of the wheat so as to induce growth and well-being. This ration is vastly improved by the inclusion of more protein (Charts 2, 6, and 8). Y marks the birth of young.

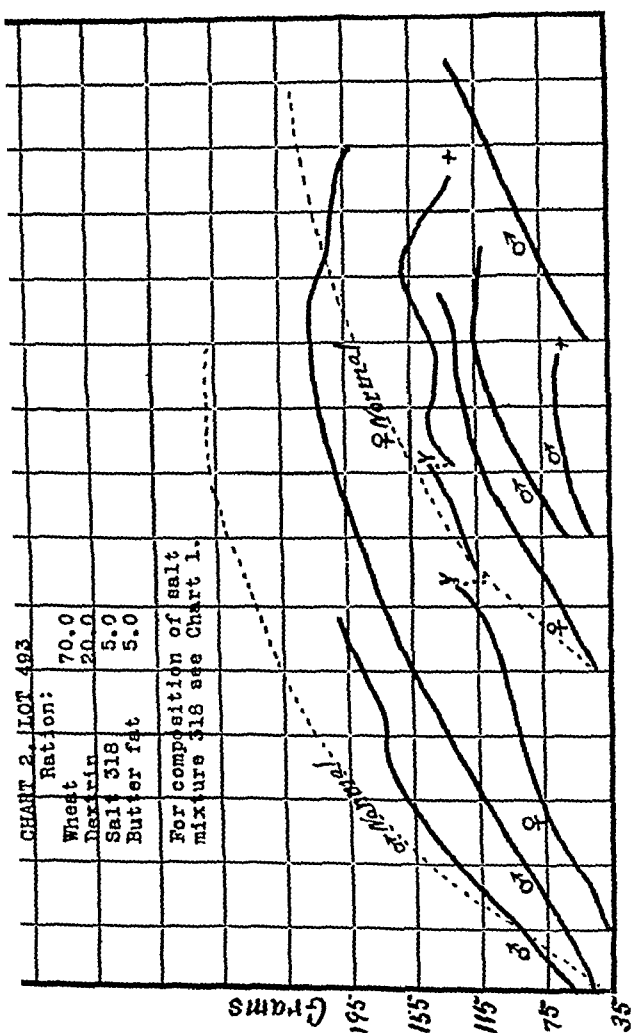


CHART 2. Lot 493 illustrates the great difference in the rate of growth of rats receiving 7.7 per cent of protein as compared with those receiving but 6.5 per cent (Chart 1). The normal adult size is never attained, however, and the span of life is short. Rats on this diet show marked signs of age at 10 to 12 months, and die at about that age. The span of life for the rat when fed a mixed diet is about 36 months. With the exception of the amount and character of the protein this food mixture is entirely adequate for complete growth and well-being (Charts 1, 6, and 8).

The two litters of young produced by one of the females in this group were not kept alive by the mother beyond the 2nd day.

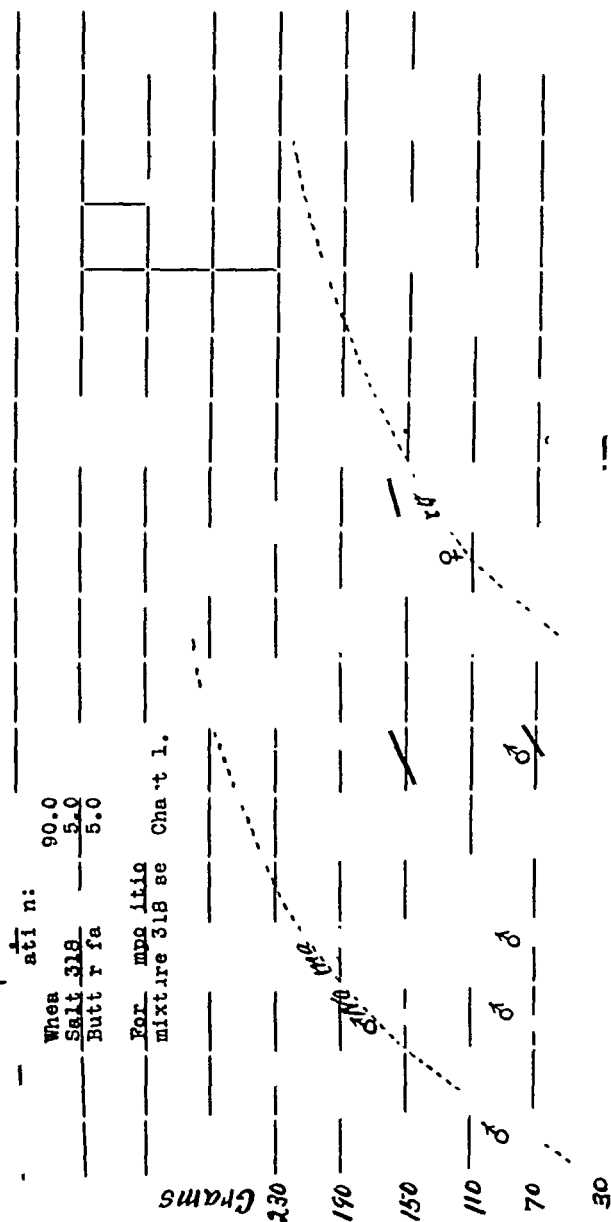


CHART 3. Lot 464 shows growth of rats at somewhat less than the normal rate, and failure to attain the full adult size on a ration otherwise satisfactory but containing only 9.9 per cent of protein, all of which was supplied by whole wheat. This amount of protein derived from the wheat *germ* is adequate for growth at the maximum possible rate. The addition of 2.2 per cent of protein over that of Lot 493 (Chart 2) did not improve the appearance or size to the degree which one might expect.

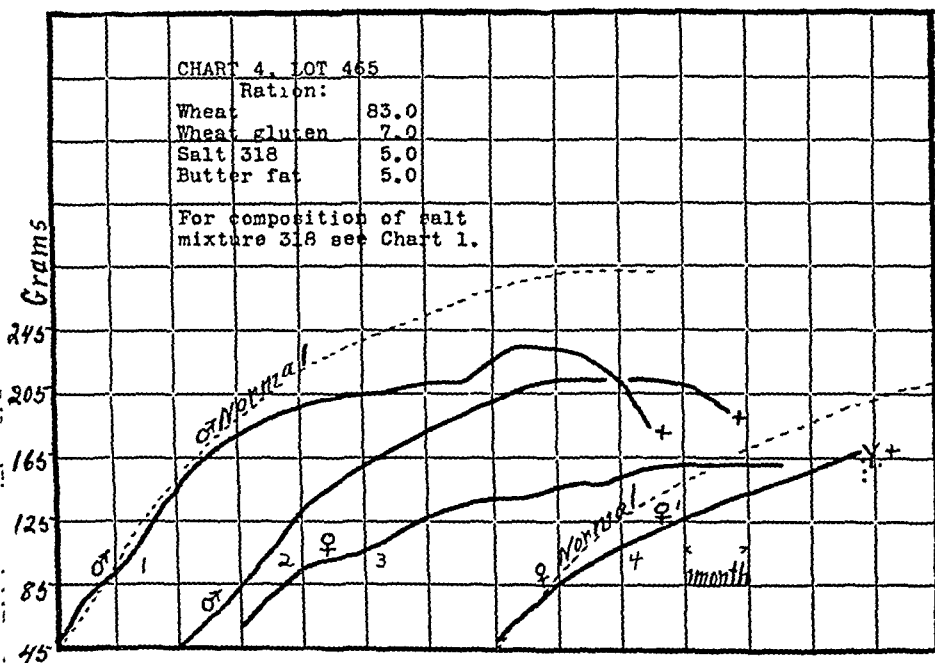


CHART 4. Lot 465 shows the surprising result that there is no improvement in growth on a food mixture closely similar to that of Lots 493 and 464 (Charts 2 and 3) except that it contained 14.45 per cent of protein instead of the 7.7 per cent and 9.9 per cent in the case of the two first mentioned lots. Here again growth fell somewhat below the normal rate and the animals were permanently stunted after having reached a size about 80 per cent as great as the normal adult. The span of life was but 9 to 10 months. The easily observable improvement which resulted in raising the wheat content from 70 to 90 per cent of the diet was not furthered by increasing the protein content to 14.45 per cent by the addition of 7 per cent of wheat gluten.

Rat 4 died at the time of the birth of two young.

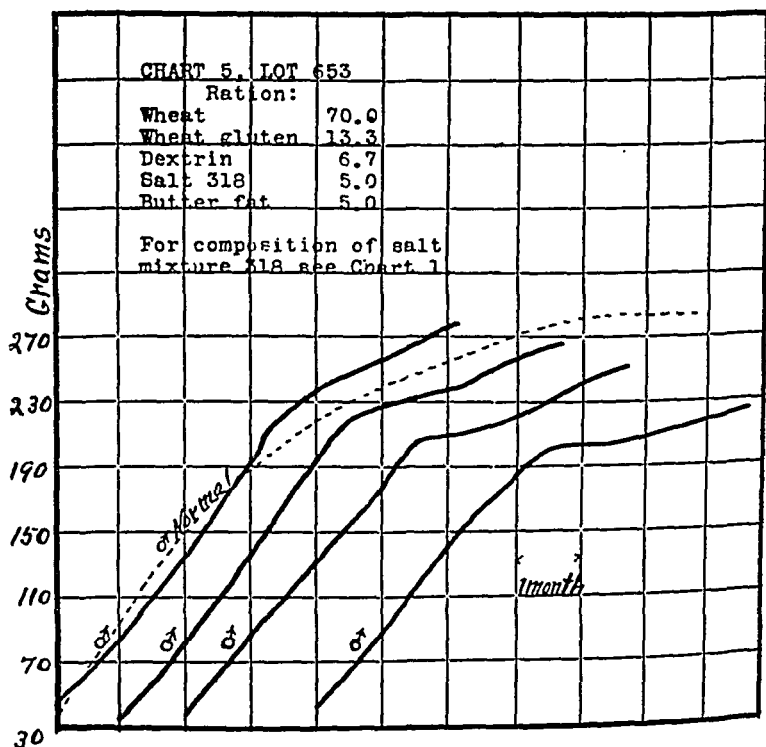


CHART 5. Lot 653, whose ration was entirely comparable to those previously described but contained 13.3 per cent of wheat gluten which raised the protein content to 17.8 per cent, grew at the normal rate to the full adult size, and were exceptionally fine-looking animals.

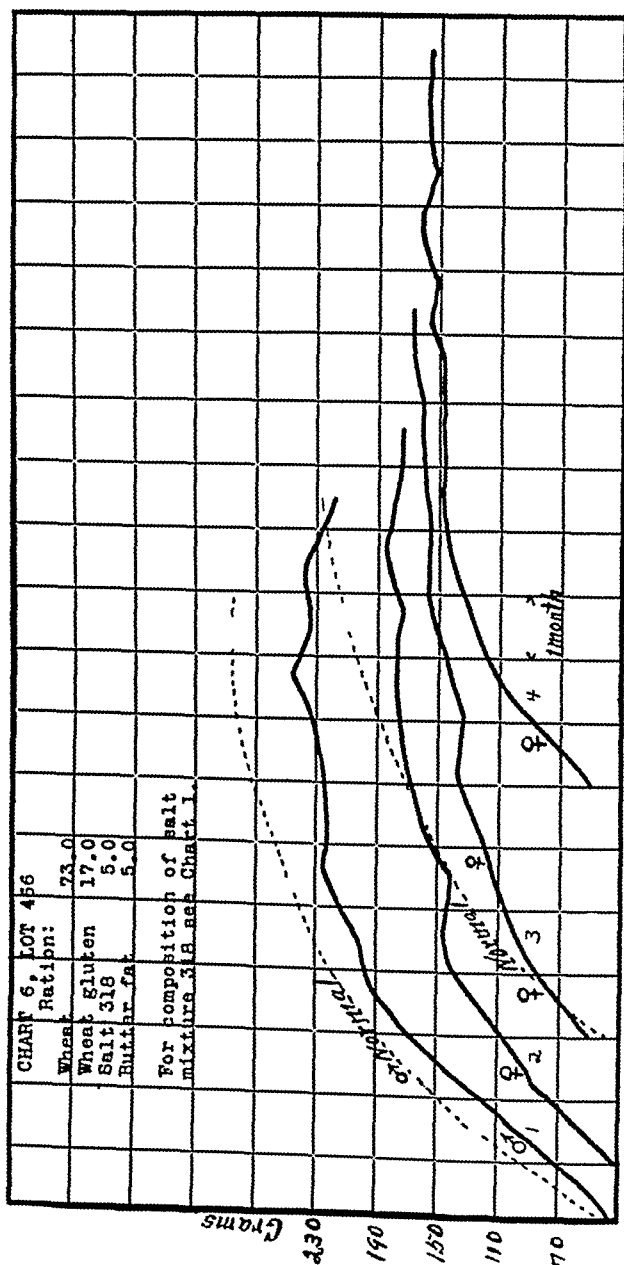


CHART 6. Lot 466, whose ration contained 20.95 per cent of wheat proteins led to stunting in some degree and none of the three females in this lot produced young, although they were kept through practically their entire period of fertility. These rats were all somewhat undersized, but one female (No. 2) reached a weight of 180 gm. Many female rats never grow larger than this.

Although the rats grew to a larger size in Lot 653 (Chart 5) with 13.3 per cent of wheat gluten in the diet, it does not seem probable that they were any better off with this lower level than were the rats receiving 17 per cent of wheat gluten. This is supported further by the fact that a still higher level of wheat gluten in Lot 467 (Chart 7) improved the nutrition of the animals sufficiently to lead to the production of nine litters of young by four females. One of them was an inferior animal and produced no young.

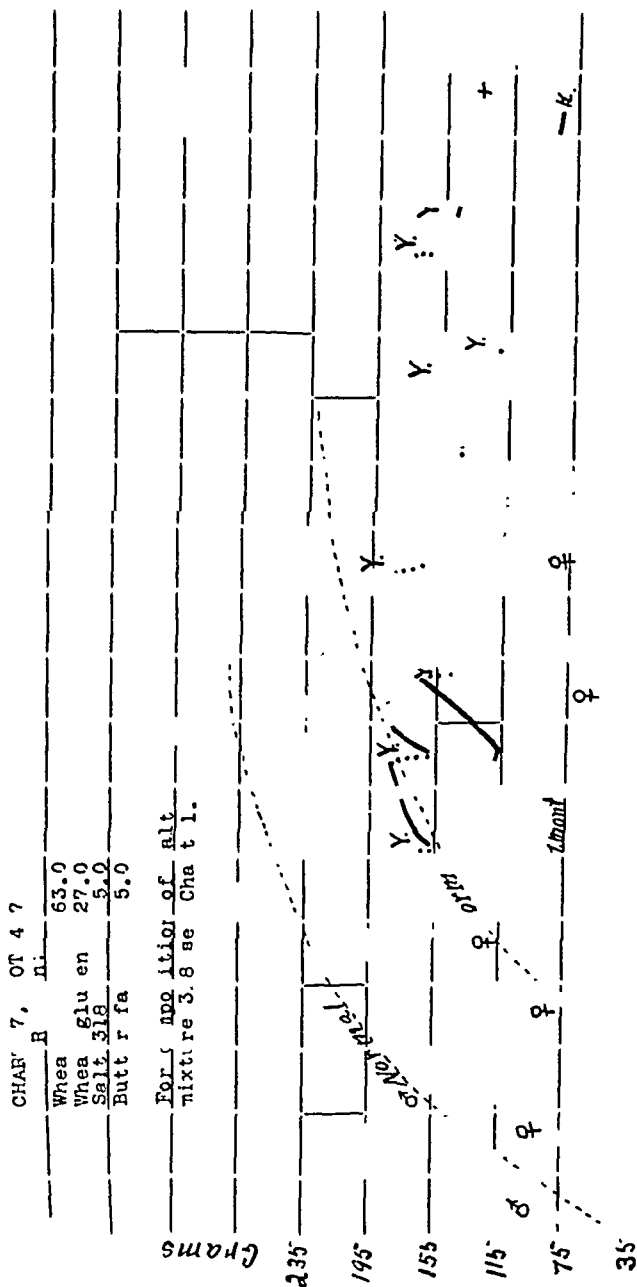


CHART 7. Lot 467, whose ration carried 27.45 per cent of protein, fell somewhat short of the normal amount of growth, but the ration induced reproduction much better than any of the rations previously described, all of which weaned; nearly all of them were allowed to die within the first 2 or 3 days after birth. One litter was brought to weaning age and at nearly the normal rate (three young, weight 65 gm., at 26 days), but they were then killed and eaten by the mother.

In all our extensive experimentation with rations restricted to wheat there has been failure to produce viable young but none have been produced by the ration described in Chart 7.

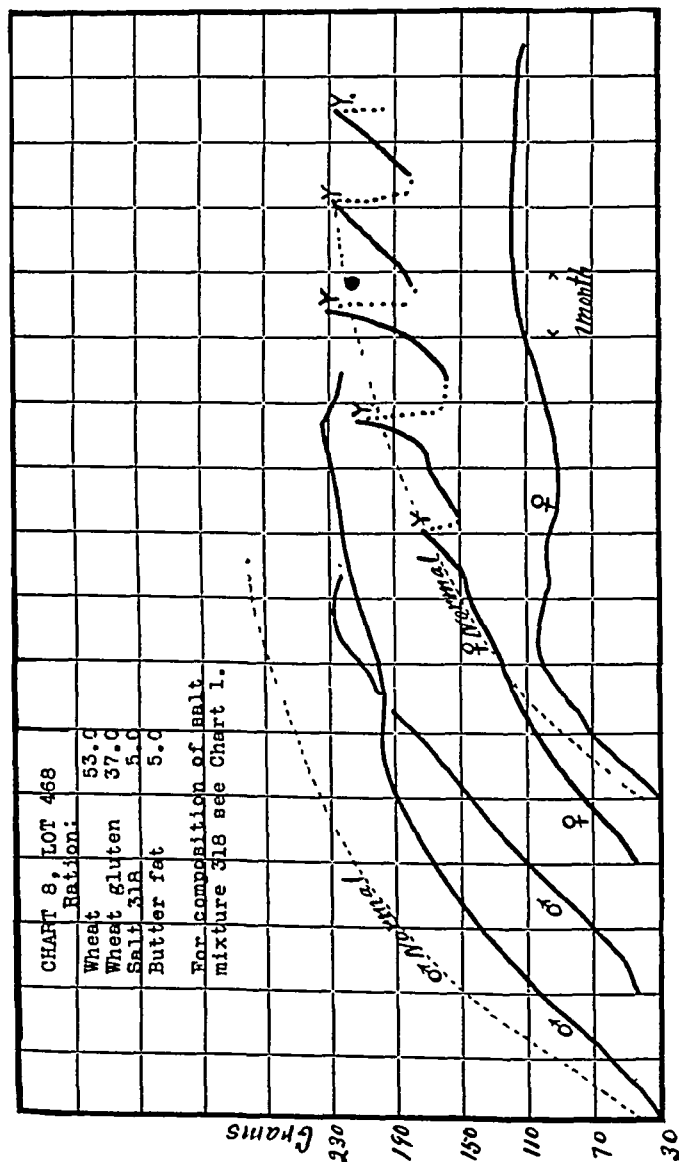


CHART 8. Lot 468. This ration contained 33.95 per cent of protein all derived from a mixture of wheat and wheat gluten. The two males never attained the full adult size, but one female produced five litters of young. She was apparently somewhat undersized. None of the young lived beyond the 14th day, and most of them died very early. Possibly two factors operate in interfering with nutrition in such a diet; viz., the presence of a toxic constituent in wheat products, and injury due to the metabolizing of a very high protein diet over an extended period. Charts 11, 12, 13, and 15 furnish data relative to the ability of the rat to tolerate surprisingly high protein intake during growth. Lot 598 (Chart 12), whose ration contained 36.33 per cent of wheat proteins and 10 per cent of casein, were able to rear a fair percentage of their young.



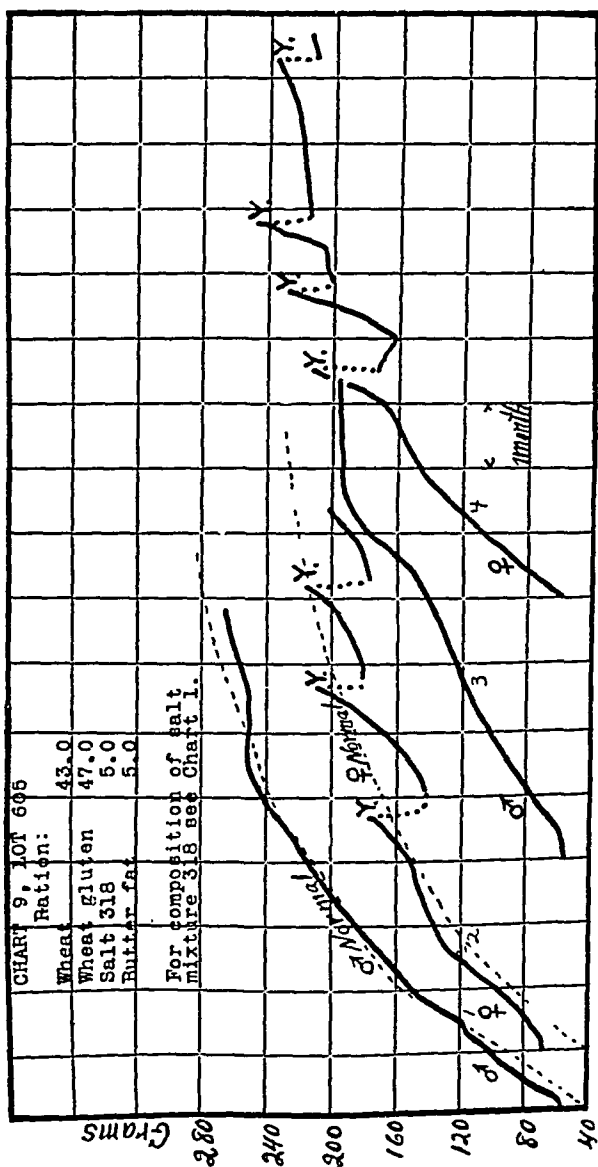


CHART 9. Lot 605 illustrates the attainment of normal growth and the production of three and four litters of young respectively by two females in this group on a diet containing 40.45 per cent of protein. Better success was attained in the rearing of the young on this diet than on any of the others described in this paper, in which all the protein came from wheat (Charts 8, 9, and 10).

The most successful instance was in the third litter of Rat 2: three of her young at 27 days of age weighed 17, 18, and 19 gm., respectively. They appeared normal except for their diminutive size.

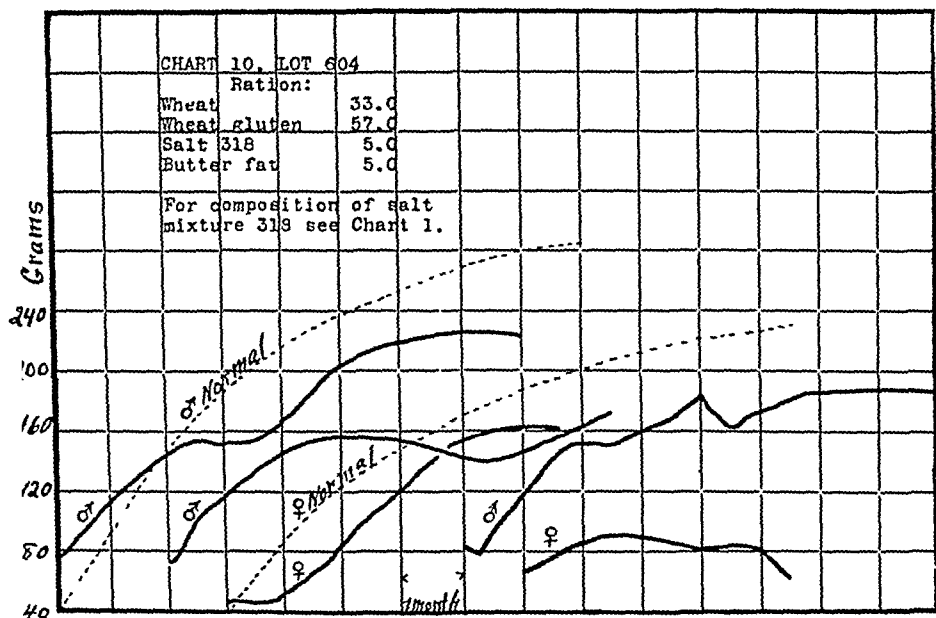


CHART 10. Lot 604, whose diet contained 47.98 per cent of protein, all derived from wheat and wheat gluten, failed to grow at the normal rate. That the failure to grow was not due to the high protein content *per se* is evident from the record of Lot 669 (Chart 15) whose ration contained 46.63 per cent of protein, 43 per cent of which was casein, yet they suffered no depression in growth and must have been in fairly good nutritive condition, since one female gave birth to four litters of young. It is the high content of the wheat proteins, and their peculiar character or a toxic substance accompanying them that causes injury to the animals. Whether or not the injury results from the metabolism of a large amount of proteins of poor quality, when the same intake of proteins yielding better proportions of cleavage products would be without harm cannot be decided from the data now available. Proteins of poor quality from several sources must be fed at high planes of intake in order to furnish an answer to this question.

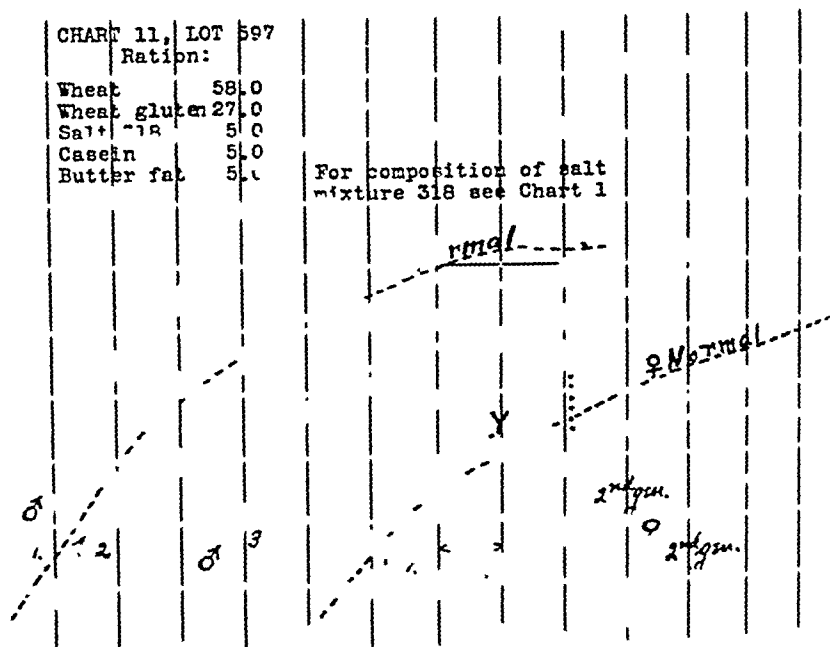


CHART 11. Lot 597 illustrates the normal growth of rats receiving 31.90 per cent of protein in their diet, 26.9 per cent being derived from wheat and wheat gluten and 5.0 per cent as casein. Female 4 produced two litters of young; the second litter of eight young was reduced to four; and in these she induced growth to a collective weight of 97 gm. at the age of 18 days. Two of these during the next 2 days passed into a condition of stupor and died. The other two were successfully weaned. Their curves of growth and the mother's diet are shown in the chart. There is little doubt that the addition of 5 per cent of casein to this ration improved it in its power of promoting growth and of admitting the rearing of young (compare with Chart 7).



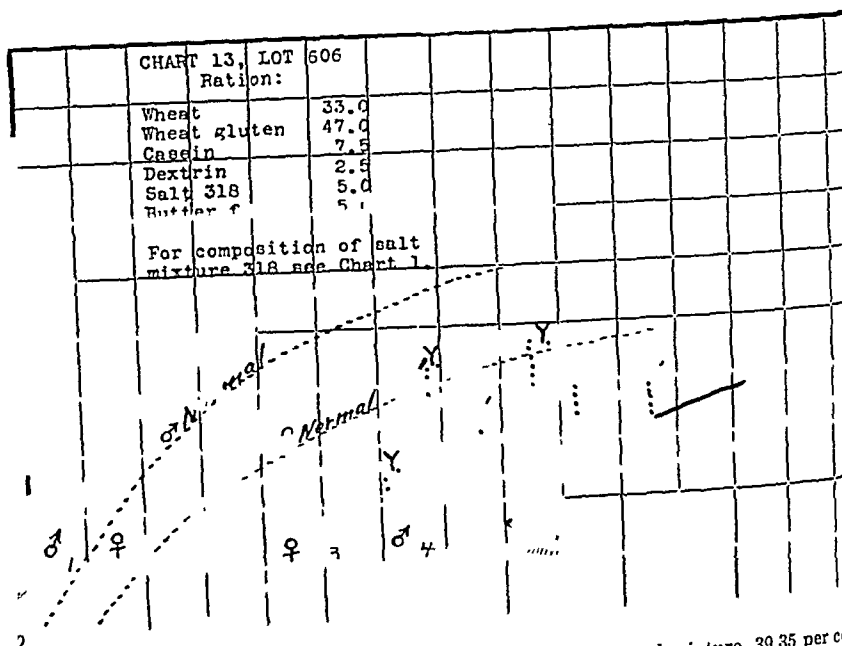


CHART 13. Lot 606 received 46.85 per cent of protein in the food mixture, 39.35 per cent which was derived from wheat and 7.5 per cent from casein. The two females produced and four litters of young respectively, of which none were weaned. Rat 3 brought of her second litter of five to the age of 20 days, when they weighed collectively 33 gm., half the normal weight for this age. The inability of the mother to induce growth in young is in marked contrast to the records of Lot 597 on the one hand, which received 10 per cent less of wheat gluten and but 5 per cent of casein, and of Lot 598 on the other whose ration contained 46.35 per cent of protein, 10 per cent of which was casein. The simultaneous raising of both casein and wheat gluten, the latter in greater relative proportion, leads to depression of the power to induce growth in the young.

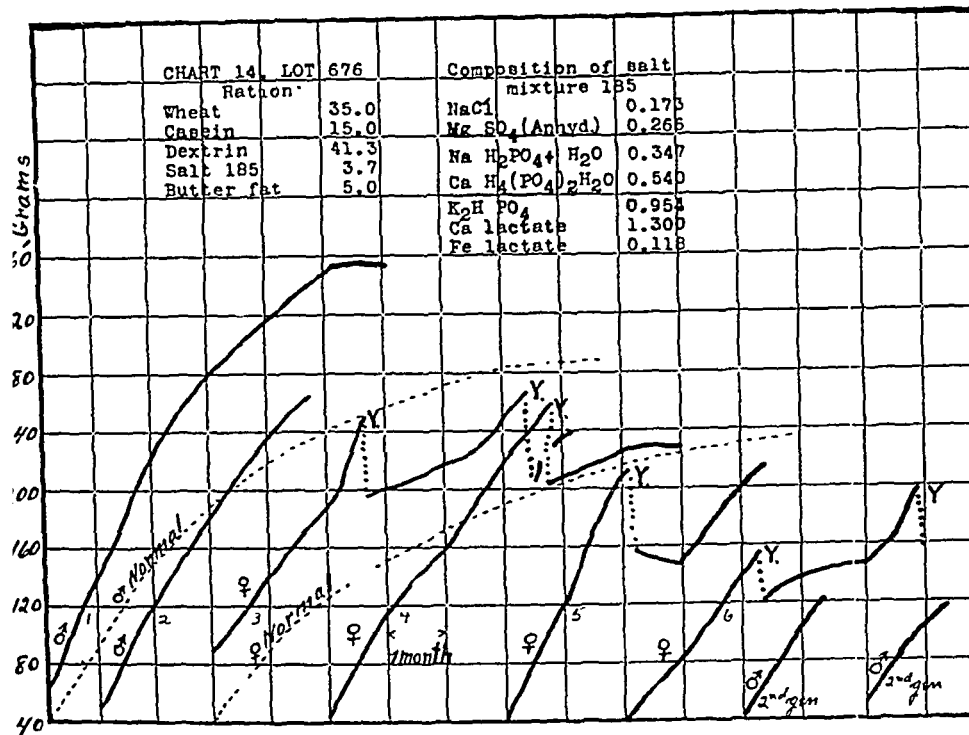


CHART 14. Lot 676 demonstrates the fact that wheat contains a great abundance of the water soluble B. This ration derived all its content of this dietary factor from its content of 35 per cent of wheat. The growth on this ration was extremely rapid and the reproduction records were good but the mortality of the young was high. There were but three weaned out of fifty young born. The cause of this failure appears to be the low content of the factor B, furnished by 35 per cent of wheat. The demand for this factor by a growing litter of young is high. Once the young were weaned, the ration, without the addition of more B, was adequate for the growth of the second generation, as is shown by the two curves of the second generation on the chart.

Without reproduction records this ration would have appeared to be near the optimum respect to every factor. With reproduction records, the limiting factor becomes apparent. This is likewise true for Lots 669 and 645 (Charts 15 and 17) respectively.



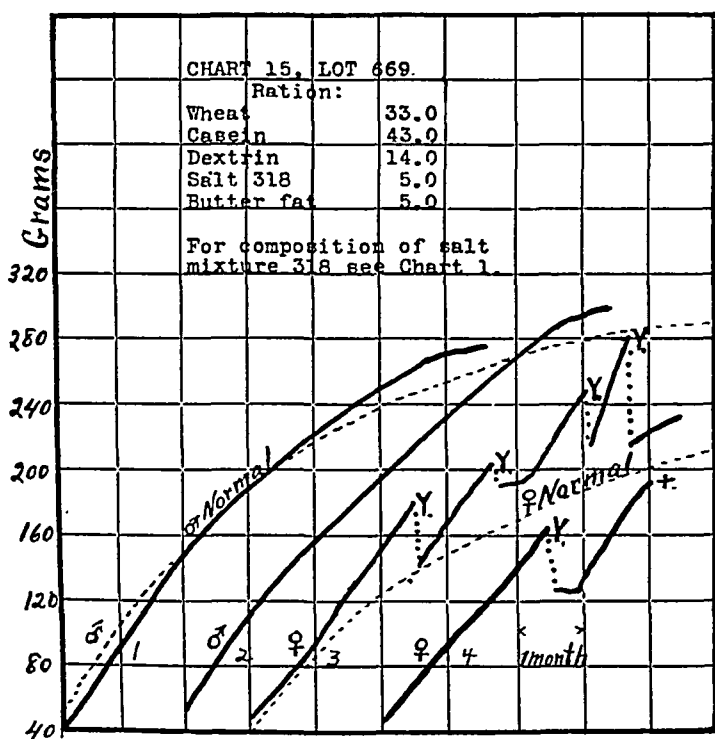


CHART 15. Lot 669 shows that growth is not interfered with by the presence of a high protein content in the diet. This ration contained 46.63 per cent of protein, 43 per cent being casein and 3.63 per cent wheat proteins. They were unable to rear young on this diet, but this may be due to the inadequate supply of the dietary B (Chart 14), all of which was furnished by 33 per cent of wheat.

Rat 4 brought five young from a weight of 28 gm. at birth to 88 gm. at the age of 14 days. 2 days later all were dead. The young from this group passed into a state of stupor before death. This condition would appear to be the result of the shortage of the water-soluble B in the milk. Since extracts containing the factor B cure polyneuritis, these results suggest that the young died of beri-beri. We have elsewhere shown that the dietary factor B is not present in the milk except when it is supplied to the mother in the diet.<sup>5</sup>

<sup>5</sup> McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxvii, 33.





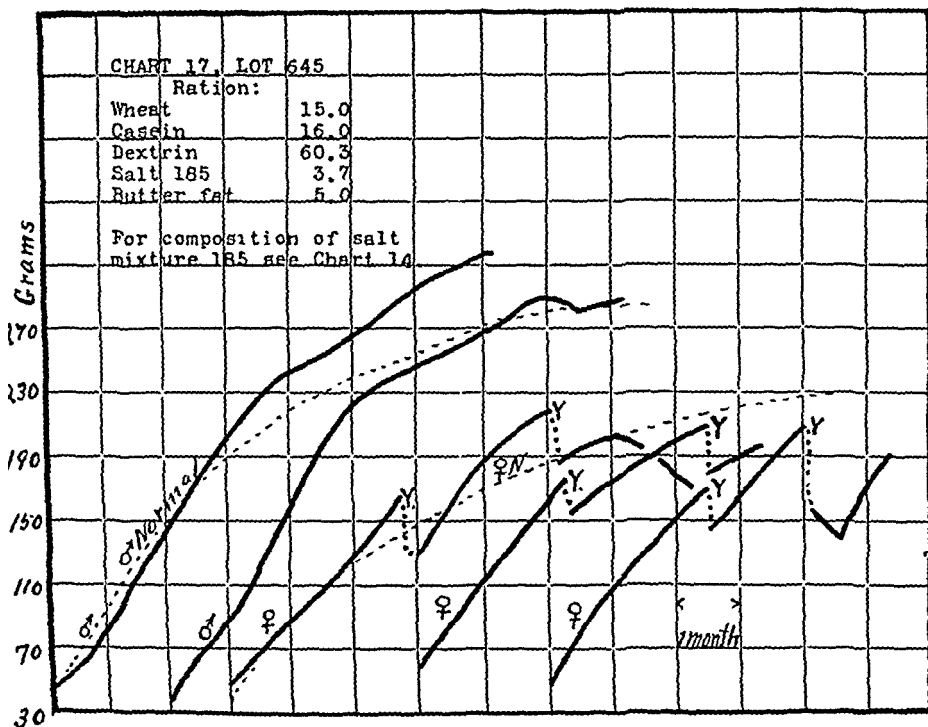


CHART 17. Lot 645 emphasizes the fact that the amount of a whole grain necessary to furnish an adequate amount of the water-soluble B to induce perfect growth and well-being is surprisingly small. This dietary factor was here supplied by 15 per cent of wheat and not only was growth normal, but the reproduction records were good. No young were reared on this diet, although in one instance a litter of five young died between the 15th and 20th days. The young that lived to this age appeared normal until a day or two before death. They then showed periods of excitement, when they would dash about the cage and would at intervals sit up on their hind legs, and gave the impression of having intense muscular spasms. After a short interval of such behavior, they became exhausted and a period of inactivity followed. The young which behaved in this fashion were apparently in a well nourished state. Three young in one litter from this group weighed 18 gm. at birth, and at the age of 9 days they weighed collectively 52 gm. At the age of 16 days these young died. Evidently lack of milk production by the mother was not the cause of the disturbance in the young. Only during the last one-half to one-third of the suckling period did the young behave abnormally. What has been said here respecting the development and behavior of the young applies also to Lots 676 and 475 (Charts 14 and 16) respectively.



# THE URINARY AND FECAL OUTPUT OF CALCIUM IN NORMAL MEN TOGETHER WITH OBSERVATIONS ON THE HYDROGEN ION CONCENTRATION OF URINE AND FECES.

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Numerous investigators, among whom we may mention Bertram,<sup>1</sup> Renvall,<sup>2</sup> von Wendt,<sup>3</sup> Sherman, Mettler, and Sinclair,<sup>4</sup> and McCrudden and Fales,<sup>5</sup> have reported on the urinary and fecal output of calcium in normal human beings. Bertram found in a 3 day period 0.167 gm. of CaO in the urine and 0.233 gm. in the feces. Renvall found in one period in an individual of 71.1 kg. body weight 0.507 gm. of CaO in the urine and 0.325 gm. in the feces. Sherman, Mettler, and Sinclair in six metabolism experiments with special diets report daily urinary values for calcium varying from 0.054 to 0.307 gm. and fecal outputs from 0.480 to 1.88 gm. McCrudden and Fales found in a normal boy weighing 27.2 kg. an average daily output of 0.3805 gm. of CaO in the urine and 1.699 gm. in the feces.

Variations in normal values, however large or small they may be, are never fortuitous, but depend on definite factors and sets of conditions. Just what these are in the case of calcium and what their relative importance is we cannot say at present, largely because enough normal data are not available. To say that calcium excretion depends on the composition of the foods ingested and that the amount absorbed varies in general with the

<sup>1</sup> Bertram, J., *Z. Biol.*, 1878, xiv, 354.

<sup>2</sup> Renvall, G., *Skand. Arch. Physiol.*, 1904, xvi, 94.

<sup>3</sup> Von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211.

<sup>4</sup> Sherman, H. C., Mettler, H. A., and Sinclair, J. E., *U. S. Dept. Agric., Office Exp. Stations, Bull.* 227, 1910.

<sup>5</sup> McCrudden, F. H., and Fales, H. L., *J. Exp. Med.*, 1912, xv, 450.

amount of acid constituents present in the food or with acid produced or introduced into the body, disposes of the question too lightly. There are undoubtedly other important factors which further accumulation of normal data will bring to light. It would be fortunate if calcium metabolism would receive as much attention as basal metabolism has lately received at the hands of Benedict and his coworkers. It seems axiomatic also that if we would know more about such diseases as osteomalacia, rickets, osteoporosis, and infantilism, we must first have forthcoming sufficient data on the normal behavior of the body, with which to compare the findings in these pathological states.

Most of the data now available on the normal urinary and fecal outputs of calcium have come from equilibrium experiments. To let an individual eat what he wants and whenever he wants cannot well be done when the amount of ingested food must be measured, so most experiments necessarily represent values on more or less restricted diets, particularly regarding vegetables, and are therefore not indicative of normal values in the best sense of the word. We have thought, therefore, that it might be worth while to report on the urinary and fecal calcium excretion under conditions where no such restrictions were made. While we have thus not been able to determine the calcium balance, the values recorded represent the actual amounts excreted under conditions which obtain in every day life, and must therefore be considered in discussing normal outputs. We have also measured the hydrogen ion concentration of the urine and feces, since these values appear to be especially significant in determining the ratio of the urinary to fecal excretion of calcium.

#### EXPERIMENTAL.

The subjects were four healthy men and one healthy boy, 22, 25, 34, 70, and 13 years of age, respectively. The urinary and fecal outputs were measured in each case over periods of 5 days.

The urine and feces were in each case carefully collected in 24 hour periods, the feces in small granite pails with tight covers. Both the urine and feces were kept in the refrigerator until analyzed. Analyses were made daily, usually begun half an hour after the 24 hour samples were collected. The calcium, both

urinary and fecal, was determined by the turbidimetric method recently reported by Lyman.<sup>6</sup> Duplicate and triplicate samples were found to agree closely if care was taken while reading the colorimeter to place it in a good north light and well above the line of trees. The hydrogen ion concentrations were determined by the Sørensen indicator method as modified by Henderson and Palmer.<sup>7</sup> For the hydrogen ion concentration of feces we have followed the plan suggested by Howe and Hawk<sup>8</sup> of using exactly 2 gm. of moist feces to 50 cc. of liquid. 10 cc. of the fecal extract thus prepared, after being thoroughly centrifuged and twice filtered, were compared with diluted standards after addition of the suitable indicator. We were able, contrary to the experience of Howe and Hawk, to prepare fecal extracts practically as clear and very little, if any, darker in color than our samples of urine.

TABLE I.

Date.	Urine.			Moist feces.			Total calcium.
	Volume.	Calcium.	P <sub>H</sub>	Amount.	Calcium.	P <sub>H</sub>	
<i>I. Normal Man, J. L. W., Age 25, Weight 56 Kg.</i>							
	cc.	gm.		gm.	gm.		gm.
Nov. 15.....	755	0.2106		80.9	0.4595		0.6701
" 16.....	760	0.1824		109.9	0.6266		0.8090
" 17.....	965	0.2373		159.5	0.9175		1.1558
" 18.....	755	0.1766		17.6	0.1114		0.2880
" 19.....	725	0.1866		138.0	0.8359		1.0225
Average...	792	0.1987		101.2	0.5902		0.7889
<i>II. Normal Man, O. H. B., Age 22, Weight 79 Kg.</i>							
Feb. 6.....	1,170	0.4129		175.1	0.9307		1.3436
" 7.....	1,180	0.4452		None.			0.4452
" 8.....	330	0.1286		160.3	0.8793		1.0079
" 9.....	1,250	0.4199		None.			0.4199
" 10.....	1,405	0.5438		161.5	0.8662		1.4100
Average...	1,067	0.3901		99.4	0.5352		0.9253

<sup>6</sup> Lyman, H., *J. Biol. Chem.*, 1915, xxi, 551.

<sup>7</sup> Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912-13, xiii, 393.

<sup>8</sup> Howe, P. E., and Hawk, P. B., *J. Biol. Chem.* 1912, xi, 129.

TABLE I—Concluded.

Date.	Urine.			Moist feces.			Total calcium.
	Volume.	Calcium.	P <sub>H</sub>	Amount.	Calcium.	P <sub>H</sub>	

## III. Normal Man, J. L. W., Age 25, Weight 56 Kg.

	cc.	gm.		gm.	gm.		gm.
Mar. 2.....	805	0.2611	6.07	30.3	0.1645	6.00	0.4256
" 3.....	770	0.2581	5.40	123.9	0.6472	5.90	0.9053
" 4.....	760	0.2533	5.40	207.5	1.1877	5.50	1.4410
" 5.....	695	0.2096	4.84	197.1	1.0053	5.40	1.2149
" 6.....	700	0.2165	5.50	92.8	0.4895	5.50	0.7060
Average...	746	0.2397	5.40	130.3	0.6988	5.66	0.9385

## IV. Normal Man, C. F. N., Age 34, Weight 61 Kg.

Feb. 14.....	1,085	0.4282	5.70	134.1	0.6548	6.00	1.0830
" 15.....	860	0.2931	6.00	154.0	0.7994	5.70	1.0925
" 16.....	1,560	0.6499	5.70	39.1	0.1893	5.70	0.8392
" 17.....	1,270	0.4118	7.48	149.2	0.7507	7.38	1.1625
" 18.....	1,165	0.4368	7.38	100.4	0.4669	5.70	0.9037
Average...	1,188	0.4419	6.43	115.3	0.5722	6.09	1.0161

## V. Normal Man, C. F. N., Age 34, Weight 61 Kg.

2,000 Cc. of Distilled Water Ingested Daily in Addition to That Taken with Meals.

Mar. 13.....	2,050	0.3203	6.50	145.5	0.7694	6.00	1.0897
" 14.....	2,085	0.3096	6.50	44.0	0.2215	5.70	0.5311
" 15.....	2,700	0.3970	6.23	92.0	0.4519	6.04	0.8489
" 16.....	2,400	0.3529	7.14	40.4	0.1879	5.93	0.5408
" 17.....	2,635	0.4250	6.80	93.4	0.4386	5.85	0.8636
Average...	2,374	0.3609	6.63	83.1	0.4139	5.90	0.7748

## VI. Normal Man, L. E. S., Age 70, Weight 58.5 Kg.

Feb. 21.....	970	0.3306	5.70	129.0	0.6601	6.70	0.9907
" 22.....	1,020	0.3399	6.90	50.4	0.2359	6.90	0.5758
" 23.....	910	0.2919	5.30	23.8	0.1109	6.70	0.4028
" 24.....	935	0.2921	5.70	105.1	0.5350	6.70	0.8271
" 25.....	800	0.2474	4.90	65.8	0.3331	6.30	0.5805
Average...	927	0.3006	5.70	74.8	0.3750	6.66	0.6756

## VII. Normal Boy, H. H., Age 13, Weight 39 Kg.

Feb. 26.....	480	0.0727	4.90	96.4	0.4587	5.30	0.5314
" 27.....	450	0.1364	4.90	1.1	0.0290	5.30	0.1654
" 28.....	670	0.1990	4.70	38.6	0.2090	5.70	0.4080
" 29.....	675	0.0983	4.75	133.7	0.7251	5.50	0.8234
Mar. 1.....	440	0.1199	5.30	7.4	0.0519	5.10	0.1718
Average...	543	0.1253	4.91	55.4	0.2947	5.38	0.4200

## DISCUSSION.

It will be seen from the results in the preceding table that the average daily output of calcium in urine and feces varied, in the cases studied, from 0.420 to 1.016 gm. (CaO 0.588 to 1.42 gm.). These results are higher than those reported by most European investigators, although they fall well within the limits of the amounts obtained by investigators in this country. Since the individuals examined were apparently in the best of health, of ages from 13 to 70 years, and furthermore, since they were all on absolutely unrestricted diets, guided solely by appetite, the results obtained should, we feel, represent normal values in calcium excretion. In the two instances where data were obtained from the same individual at different periods (J. L. W., Nov. 15 and Mar. 2, and C. F. N., Feb. 14, and Mar. 13) the difference in the average daily output was 0.15 and 0.24 gm. of calcium (0.21 to 0.34 gm. of CaO), respectively. From subsequent data from the same individuals covering four additional 5 day periods during an interval of more than 6 months, these differences remained practically within the same limits. The daily combined urinary and fecal excretion of calcium, on unrestricted diets, may thus be said to vary roughly from 15 to 25 per cent.

Theoretically, at least, the relative acidity or alkalinity of the urine should to some extent determine the amount of lime that would leave the body through this channel. We know of no other determination of normal calcium outputs where this value has been determined, and since the data presented in this paper are hardly sufficient to draw safe conclusions of a positive or negative nature along these lines, we merely record the H ion concentration values for future reference.

## SUMMARY.

The average daily urinary and fecal output of calcium calculated as oxide over periods of 5 days for five apparently normal individuals, ranging from 13 to 70 years, shows extreme daily variations as follows:



In the remaining eight cases the magnesium predominated. It seems, therefore, that either calcium or magnesium may be excreted in the larger amount in the healthy individual, and that

TABLE I.

Day.	Volume.	CaO	MgO
<i>I. W. E. B.</i>			
	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>
1	1,340	0.1266	0.2950
2	1,660	0.2315	0.3706
3	1,100	0.1452	0.2215
4	985	0.1503	0.4160
5	1,400	0.1645	0.2620
Average.....	1,297	0.1636	0.3130
<i>II. W. E. B. 2,200 Cc. of Distilled Water Ingested Daily.</i>			
1	2,600	0.1839	0.2258
2	2,300	0.1247	0.1895
3	2,360	0.1298	0.2015
4	2,280	0.1368	0.1900
Average.....	2,385	0.1438	0.2017
<i>III. C. F. N.</i>			
1	1,520	.1413	0.2350
2	1,450	0.1961	0.2210
3	1,320	0.2257	0.2160
4	1,170	0.1544	0.1480
5	1,190	0.1250	0.1947
Average.....	1,330	0.1685	0.2029
<i>IV. H. S. O.</i>			
1	1,530	0.1208	0.1544
2	1,290	0.1573	0.2190
3	1,310	0.1375	0.1650
4	1,330	0.2196	0.2240
5	1,320	0.0990	0.1938
Average.....	1,356	0.1468	0.1912

the preponderance of one element is as indicative of normal behavior as the other. Furthermore, our results seem to indicate that any given individual exhibits a constant behavior as to

whether calcium or magnesium shall be greater in amount in the urine voided. In the case of the three individuals examined over the 5 day periods, we found in nineteen determinations only two in which the daily output of calcium exceeded that of magnesium. We have subsequently measured the urinary calcium and magnesium output of two of the same three individuals in connection

TABLE II.

*Daily Urinary Output of Twenty-Two Apparently Normal University Students.*

Volume.	CaO	MgO
cc	gm.	gm.
2,500	0.4875	0.3029
1,540	0.4658	0.1998
685	0.3905	0.2630
1,420	0.3727	0.2431
960	0.3403	0.2235
1,030	0.3347	0.2340
680	0.3097	0.1180
610	0.2822	0.1400
650	0.2707	0.2340
880	0.2662	0.1353
1,920	0.2582	0.1625
1,980	0.1989	0.1795
1,050	0.1916	0.1540
640	0.1891	0.1705
1,570	0.1734	0.1582
700	0.1366	0.1252
1,620	0.1255	0.1200
1,130	0.2423	0.2800
700	0.2310	0.2472
910	0.1915	0.2260
785	0.1790	0.2115
1,120	0.1260	0.1353

with other experiments over two 5 day periods and three 3 day periods, and we have found again in nineteen determinations only two in which calcium predominated. The interval between the first and last of these experiments was fully 4 months, a period which seems long enough to eliminate effectually the possibility of the constant preponderance of the magnesium being due to a sameness of diet.

Maillard,<sup>5</sup> Gortner, Gortner and Blish,<sup>6</sup> and Roxas,<sup>7</sup> confirmed the view that nitrogen-containing humin will be formed when certain amino-acids and carbohydrates are heated together in contact with acids, or even, as Maillard showed, in water solutions at temperatures of 100–150°.

Gortner and Blish in their early work contended that tryptophane alone among the amino-acids was responsible for humin formation. Their conclusions were as follows: The humin nitrogen belongs to no amino-acids other than tryptophane. The reaction involved is probably due to the condensation of the aldehyde with the  $-NH$  group of the tryptophane nucleus. Histidine can be eliminated as a factor in the formation of humin nitrogen.

Gortner has since modified some of these views. Recently Roxas,<sup>7</sup> working in this laboratory on humin formation with definite carbohydrates and amino-acids, reached the following conclusions:

"1. Alanine, leucine, phenylalanine, and glutaminic acid may be eliminated as important factors in humin formation when subjected to the treatment used in these experiments. Proline, however, under certain conditions, may be involved in humin formation.

"2. The following amino-acids are responsible for humin formation and in digestions with 20 per cent HCl plus sugar, the proportion of their nitrogen disappearing was: Tyrosine, 15.0; cystine, 3.1; arginine, 2.33; lysine, 2.62; histidine, 1.84; and tryptophane 71.0.

"3. Xylose and fructose were, as a rule, more reactive than glucose.

"4. Arginine, histidine, and lysine reacted with sugars more readily in weak acids or aqueous, than in strong acid solutions.

"5. Arginine, histidine, and tryptophane reacted with loss in reactivity of their amino nitrogen towards nitrous acid, but tyrosine and cystine reacted without any such loss."

Roxas has, therefore, specifically demonstrated that the hexone bases, as well as tryptophane and tyrosine, are to be considered as taking part in humin formation. The amount of humin formation will depend upon the nature and quantity of the carbohydrate present in the hydrolyzing solution.

Roxas worked on individual amino-acids in the presence of different carbohydrates. However, his experiments with proline and cystine showed that the reaction may be quite different when a mixture of amino-acids is present. Proline, although forming no humin nitrogen when boiled alone with glucose in a 20 per cent HCl solution, produced a decided increase in humin formation when the reaction was carried on in the presence of cystine,

<sup>5</sup> Maillard, L.-C., *Compt. rend. Acad.*, 1912, cliv, 66; *Compt. rend. Soc. biol.*, 1912, lxxii, 511.

<sup>6</sup> Gortner, R. A., *J. Biol. Chem.*, 1916, xxvi, 177. Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.

<sup>7</sup> Roxas, M. L., *J. Biol. Chem.*, 1916, xxvii, 71.

over what cystine alone would produce. It was, therefore, of importance to investigate the effect of a mixture of amino-acids, formed by the hydrolysis of a protein in the presence of various carbohydrates, on humin formation and the accuracy of the Van Slyke method. Casein, because of the extended study given it by Van Slyke with the nitrous acid method, was used for this work.<sup>8</sup> It was hydrolyzed alone and separately with dextrose, sucrose, starch, and xylan. 6 gm. of casein were used, and five times this amount as carbohydrate in all cases with the exception of xylan, where only two and a half times the casein was used. The hydrolysis was carried on for 48 hours in 20 per cent HCl and the Van Slyke method for protein analysis was carefully followed.

The primary purpose of this research was to determine whether carbohydrates would seriously influence the accuracy of Van Slyke's method of amino-acid determination and whether its application to feedingstuffs directly, as Grindley proposes, could be justified by the results. The records of the analyses are given in Tables I to VI.

TABLE I.  
*Comparative Analyses of 2.4 Gm. of Casein Alone.*

	Nitrogen.		Per cent of total.			Van Slyke found.
	I.	II.	I.	II.	Average.	
	gm.	gm.				
Total.....	0.326	0.326				
Ammonia.....	0.03479	0.032949	10.64	10.10	10.37	10.27
Humin.....	0.00504	0.00462	1.54	1.41	1.47	1.28
N in filtrate from bases.						
Total.....	0.219528	0.21840	67.34	67.00	67.17	62.94
Amino.....	0.192958	0.194043	59.12	59.52	59.32	55.81
Non-amino.....	0.026570	0.024357	8.22	7.48	7.85	7.13
N of bases.						
Total.....	0.075264	0.078064	23.08	23.94	23.51	24.07
Amino.....	0.047100	0.044800	14.44	13.74	14.09	14.60
Non-amino.....	0.028164	0.033264	8.64	10.20	9.42	9.47
Arginine.....	0.026208	0.025648	8.03	7.86	7.95	7.41
Cystine.....	0.00064	0.00064	0.19	0.19	0.19	0.20
Histidine.....	0.017762	0.021042	5.44	6.45	5.95	5.76
Lysine.....	0.030654	0.030728	9.40	9.42	9.41	10.70
Total recovered.....	0.33462	0.33973	102.60	102.43	102.52	98.56

<sup>8</sup> Van Slyke, *J. Biol. Chem.*, 1913-14, xvi, 531.

TABLE II.  
Hydrolysis of 2.4 Gm. of Casein + 12 Gm. of Dextrose.

	Nitrogen.		Per cent of total.		
	I.	II.	I.	II.	Average.
	gm.	gm.			
Total.....	0.3252	0.3250			
Ammonia.....	0.033586	0.032060	10.32	9.83	10.09
Humin.....	0.012320	0.012180	3.78	3.74	3.76
N in filtrate from bases.					
Total.....	0.21840	0.21560	67.15	66.33	66.74
Amino.....	0.19060	0.18555	58.61	57.09	57.85
Non-amino.....	0.02780	0.03005	8.54	9.24	8.89
N of bases.					
Total.....	0.07056	0.07336	21.69	22.56	22.13
Amino.....	0.03696	0.03826	11.36	11.45	11.40
Non-amino.....	0.03360	0.03510	10.33	11.11	10.75
Arginine.....	0.024696	0.024696	7.59	7.59	7.59
Cystine.....	0.00064	0.00064	0.19	0.19	0.19
Histidine.....	0.022623	0.02487	6.95	7.67	7.31
Lysine.....	0.022600	0.023153	6.90	7.12	7.01
Total recovered.....	0.334866	0.333200	102.94	102.49	102.71

TABLE III.  
Hydrolysis of 2.4 Gm. of Casein + 12 Gm. of Sucrose.

	Nitrogen.		Per cent of total.		
	I.	II.	I.	II.	Average.
	gm.	gm.			
Total.....	0.3286	0.3286			
Ammonia.....	0.02891	0.02961	8.07	9.01	8.54
Humin.....	0.028854	0.03129	8.78	9.52	9.15
N in filtrate from bases.					
Total.....	0.21168	0.21336	64.41	64.93	64.67
Amino.....	0.183516	0.18062	55.81	51.96	55.38
Non-amino.....	0.02816	0.03274	8.60	9.97	9.28
N of bases.					
Total.....	0.06888	0.06927	20.97	21.08	21.03
Amino.....	0.036435	0.034739	11.08	10.05	10.56
Non-amino.....	0.032445	0.034531	9.89	11.03	10.46
Arginine.....	0.022288	0.022288	6.77	6.77	6.77
Cystine.....	0.00064	0.00064	0.19	0.19	0.19
Histidine.....	0.023594	0.026720	7.18	8.13	7.65
Lysine.....	0.022358	0.019622	6.80	5.96	6.38
Total recovered.....	0.33832	0.34348	102.23	104.52	103.38

TABLE IV.  
*Hydrolysis of 2.4 Gm. of Casein + 12 Gm. of Starch.*

	Nitrogen.		Per cent of total.		
	I.	II.	I.	II.	Average.
	gm.	gm.			
Total.....	0.3320	0.3320			
Ammonia.....	0.031528	0.02961	9.49	8.91	9.20
Humin.....	0.02471	0.02485	7.44	7.48	7.46
N in filtrate from bases.					
Total.....	0.213584	0.215704	64.33	64.97	64.65
Amino.....	0.168640	0.169330	50.78	51.00	50.89
Non-amino.....	0.044940	0.046374	13.55	13.97	13.76
N of bases.					
Total.....	0.06608	0.065408	19.90	19.69	19.60
Amino.....	0.03456	0.033538	10.40	10.10	10.25
Non-amino.....	0.03152	0.03187	9.50	9.59	9.55
Arginine.....	0.02184	0.02296	6.57	6.91	6.74
Cystine.....	0.00064	0.00064	0.19	0.19	0.19
Histidine.....	0.02271	0.02583	6.81	7.78	7.30
Lysine.....	0.02089	0.015978	6.28	4.81	5.54
Total recovered.....	0.335902	0.33524	101.16	101.05	101.10

TABLE V.  
*Hydrolysis of 2.4 Gm. of Casein + 6 Gm. of Xylan.*

	Nitrogen.		Per cent of total.		
	I	II.	I.	II.	Average.
	gm.	gm.			
Total.....	0.3320	0.3320			
Ammonia.....	0.03451	0.03451	10.40	10.40	10.40
Humin.....	0.04100	0.03752	12.35	11.35	11.83
N in filtrate from bases.					
Total.....	0.192752	0.197120	58.05	59.37	58.71
Amino.....	0.182155	0.183020	54.85	55.12	54.98
Non-amino.....	0.010637	0.01410	3.20	4.25	3.73
N of bases.					
Total.....	0.06479	0.066152	19.51	19.91	19.71
Amino.....	0.039206	0.039536	11.80	11.30	11.55
Non-amino.....	0.025584	0.026616	7.71	8.61	8.16
Arginine.....	0.018928	0.018032	5.70	5.43	5.56
Cystine.....	0.00064	0.00064	0.19	0.19	0.19
Histidine.....	0.017082	0.019638	5.14	5.91	5.52
Lysine.....	0.028140	0.027842	8.53	8.57	8.55
Total recovered.....	0.33305	0.33530	100.31	101.03	100.66

TABLE VI.

*Summary of Data. Nitrogen Expressed as Per Cent of Total.*

	Casein.					
	Van Slyke.	Authors.	+ Dextrose.	+ Sucrose.	+ Starch.	+ Xylan.
Ammonia.....	10.27	10.37	10.09	8.54	9.20	10.40
Humin.....	1.28	1.47	3.76	9.15	7.46	11.83
N in filtrate from bases.						
Total.....	62.94	67.17	66.74	64.67	64.65	58.71
Amino.....	55.81	59.32	57.85	55.38	50.89	54.98
Non-amino.....	7.13	7.85	8.89	9.28	13.76	3.73
N of bases.						
Total.....	24.07	23.51	22.13	21.03	19.60	19.71
Amino.....	14.60	14.09	11.40	10.56	10.25	11.55
Non-amino.....	9.47	9.42	10.75	10.46	9.55	8.16
Arginine.....	7.41	7.95	7.59	6.77	6.74	5.56
Cystine.....	0.20	0.19	0.19	0.19	0.19	0.19
Histidine.....	5.76	5.95	7.31	7.65	7.30	5.52
Lysine.....	10.70	9.41	7.01	6.38	5.54	8.55
Total recovered....	98.56	102.52	102.71	103.38	101.10	100.66

## DISCUSSION.

Compared with pure casein the ammonia nitrogen was practically the same in the case of dextrose or xylan plus casein, but there was an appreciable loss in the case of sucrose and starch, it being probably converted into humin nitrogen. It will be noted that there was a striking difference in the production of humin nitrogen. In the case of casein and dextrose the humin nitrogen was about two and one-half times that obtained with casein alone, while with casein and sucrose it was more than six times as great. This can be explained by the fact that sucrose is hydrolyzed into dextrose and levulose, and that the latter, according to the work of Roxas, is more reactive in humin formation than is glucose. In the case of the starch hydrolysis the humin nitrogen is less than in the case of the sucrose hydrolysis. This probably rests upon the fact that the main product of hydrolysis of starch is glucose. The case of casein and xylan was most interesting. With half as much carbohydrate present the amount of humin nitrogen

formed was the largest, being about eight times the amount formed when casein was treated alone. This fact also agrees with the work of Roxas, who showed that there was a greater reactivity of the pentose sugar, xylose, toward humin formation in the presence of amino-acids than shown by the hexose sugar, glucose.

The loss in all cases in the hexone bases and particularly in the amino fraction of the bases was very evident. Arginine was low in all the hydrolyses and varied considerably. About 12 per cent was lost in the hydrolysis of casein and starch and about 30 per cent in the case of casein and xylan. Histidine, however, was high in all cases with the exception of the xylan hydrolysis, where it was low. This can be explained as follows: Histidine is calculated from the definite relation between the total and amino nitrogen. The non-amino nitrogen comes from three-fourths of the arginine nitrogen and two-thirds of the histidine nitrogen. If D represents the non-amino nitrogen, which is obtained from the difference between the total and the amino nitrogen, H the histidine nitrogen, and A the arginine nitrogen, we have,

$$D = (\frac{3}{4} A + \frac{2}{3} H) \text{ or } \frac{2}{3} H = (D - \frac{3}{4} A) \text{ or } H = (D - \frac{3}{4} A) \frac{3}{2}.$$

It is apparent that any factor which will tend to increase D or diminish A will increase H. It can be seen in the table of results that there was a loss of amino nitrogen and therefore an increase of non-amino nitrogen in all cases with the exception of the xylan hydrolysis. However, the histidine nitrogen in the xylan hydrolysis, while only 0.43 per cent less than in the case of the casein hydrolysis, was considerably less than in the hydrolysis in the presence of the other carbohydrates. This is explained by the fact that the loss of amino nitrogen has not been correspondingly great. However, the loss of total nitrogen of the bases was considerably greater than in the case of dextrose and sucrose and almost the same as in the case of starch. The loss was, therefore, apparently in the non-amino nitrogen and that would make histidine low.

Lysine is obtained by the difference between the total nitrogen of the bases and the sum of the arginine, cystine, and histidine. If the sum of arginine and histidine is high, lysine will be low. If histidine is low, lysine will be high. *In the presence of starch*



there was approximately a 50 per cent error in the lysine determination.

It might be stated in this connection that Buckner, Nollau, and Kastle<sup>9</sup> have recently published some work on the relation to growth of the lysine content of different feeding mixtures, applying the Van Slyke method directly, and from such analyses have drawn some remarkable conclusions. It may be inferred from what is here presented that sweeping conclusions on nutritive efficiency, based on the lysine content of feedingstuffs determined by direct hydrolysis, cannot be accepted.

Cystine represented such a small fraction of the total nitrogen, only 0.20 per cent, that it was thought safer to use in the calculations the amount found by Van Slyke in casein hydrolysis rather than the value obtained by direct determination. In our hydrolysis the amount found was too small to make the results trustworthy.

There was practically no loss of total monoamino nitrogen in the hydrolysis of casein in the presence of dextrose, but about 3 per cent loss in the presence of sucrose or starch. However, the most striking loss occurred in the presence of xylan. There about 13 per cent of the total monoamino nitrogen disappeared.

Another interesting fact in connection with this work was the difference in the non-amino nitrogen of the filtrate from the bases. In the starch-casein hydrolysis it was 13.76 per cent of the total nitrogen and but 3.73 in the xylan-casein hydrolysis, the difference being greater than the difference in the total nitrogen of the filtrates. The explanation is probably as follows: The filtrate from the bases in the case of starch was very dark brown in color, but in the case of xylan it was light yellow. It is probable that in the presence of starch, humins were formed, which were soluble in 10 per cent calcium oxide, the base used to remove them. In that case we should have more total nitrogen in the filtrate, but less amino nitrogen, which agrees with the facts. Since the completion of this work Gortner<sup>6</sup> has obtained similar results with fibrin, hydrolyzed in the presence of various carbohydrates.

<sup>9</sup> Buckner, G. D., Nollau, E. H., and Kastle, J. H., *Am. J. Physiol.*, 1916, xxxix, 162; *Kentucky Agric. Exp. Station, Bull.* 197, 1916.

## SUMMARY.

1. The Van Slyke method of protein analysis, applied to casein, hydrolyzed in the presence of various carbohydrates, brings about a total redistribution of the amino-acids, varying with the nature of the carbohydrate employed. The redistribution is especially apparent in the hexone bases and a decided loss of monoamino nitrogen also occurs where the hydrolysis takes place in the presence of xylan.

2. This work on casein and Gortner's work on fibrin, hydrolyzed in the presence of cellulose, definitely show the inapplicability of the method of direct hydrolysis for the estimation of amino-acids in feedingstuffs by Van Slyke's method. The results so secured will be inaccurate.

3. It also appears impossible to establish factors of correction for the method, because of the variation in the nature and quantity of the carbohydrates in feedingstuffs. Because of this variation in the nature of the carbohydrate content it does not seem possible even to obtain comparative figures of any great worth.

4. These results warrant the conclusion that in the present unsatisfactory status of the methods for estimating the amino-acids in the complex protein-carbohydrate mixture of feedingstuffs, the only reliable procedure for obtaining an insight into the nutritive worth of the proteins in such a mixture will be the biological one.



# A METHOD FOR THE MEASUREMENT OF THE UREA-EXCRETING FUNCTION OF THE KIDNEYS.

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If it may be assumed that under the same conditions the same kidneys will act in the same manner, we could take the urine secreted by different individuals as a measure of the degree of difference between their kidneys, providing the conditions under which they acted were exactly the same. Leaving aside the possibility, for which we have as yet no satisfactory evidence, that there are true secretory nerves regulating kidney action, it may be said that if we could control the amount and the composition of the blood supplied to their kidneys, we should probably succeed in establishing identical experimental conditions.

Of course this is not possible. We cannot even regulate the concentration of any of the normal constituents of the blood. The blood urea concentration, for instance, has been shown to vary through a wide range in healthy individuals in spite of constant dietary conditions.<sup>1</sup>

But though we cannot reduce the blood urea concentration to constancy, we can determine the level at which it stands throughout a short period of time during which urine is collected. We then know the concentration of urea in the blood supplying the kidneys during this period, and when we have found the amount of urea in the urine, we can compare the one with the other and say that in 1 hour, for instance, the kidneys have excreted so many times more urea than there was present in 100 cc. of the blood which passed through them.

If we could assume, as we certainly cannot, that the rate of flow of blood through the kidneys is constant, this ratio would

<sup>1</sup> Addis, T., and Watanabe, C. K., *Arch. Int. Med.*, in press.

then be a measure of the proportion between the urea excreted by the kidneys and the total urea brought to them in the blood. But the ratio

$$\frac{\text{Gm. of urea in 1 hr's. urine}}{\text{Gm. of urea in 100 cc. of blood}}$$

makes no such assumption. It is at once apparent that the quantity 100 cc. of blood has been arbitrarily chosen. The true amount of blood which passes through the kidneys of man in 1 hour must obviously be much greater though we have no data by means of which we can even approximate the average amount or calculate its possible variations.

Just on that account the above mode of expression is advantageous, since it serves to remind us of at least one essential factor, which would have to be measured before any exact expression of kidney function became possible. As we shall show later, it is not the only one, for variations in the concentration in the blood of other substances besides urea may influence the rate of urea excretion.

These unmeasured factors, and possibly others of which we do not know, are of sufficient importance to render fallacious Ambard and Weill's<sup>2</sup> attempt to formulate precise laws for the rate of urea secretion on the sole basis of the concentration of urea in the blood and urine.<sup>3</sup> They were unfortunately misled by the results of an insufficient number of observations. Yet one general idea underlying their work, *i.e.*, that in the estimation of the function of the kidney in excreting any substance, the concentration of that substance in the blood should be taken into account, is a sound one, at any rate in relation to the urea-excreting function of the kidneys.

The application of this principle in the form of our ratio does not, however, in itself give a good measure of function. It is only under certain definite conditions that it becomes of value. These conditions involve three requirements: first, since the ratio varies with such controllable conditions as food and water intake, it is necessary that these should be standardized; second, since the ratio varies with still other conditions which cannot be

<sup>2</sup> Ambard, L., and Weill, A., *J. physiol. et path. gén.*, 1912, xiv, 753.

<sup>3</sup> Addis and Watanabe, *J. Biol. Chem.*, 1916, xxiv, 203.

controlled, it is necessary that means be adopted to render them at least relatively less important; and finally, since we wish to use the method as a means of detecting anatomical differences in the kidneys of different individuals, it is necessary that the test should be carried out under conditions which accentuate such differences as sharply as possible.

The first of these requirements is easily met. The second and third are fulfilled by giving a large quantity of urea before the ratio is determined.

It may be that part of the stabilizing effect of urea ingestion on the ratio results from a tendency towards an equalization of the rate of flow of blood through the kidneys under the stimulus of the sudden and considerable rise in blood urea concentration. In any case the measurable factor—the blood urea concentration—is increased, and the effect of other variables will be relatively decreased. But it is particularly in connection with the third requirement that the administration of urea is essential. The effect on function of lesser degrees of anatomical defect may only be revealed by subjecting the kidneys to the strain of a maximal call on their functional capacity. The importance of strain is illustrated in the experiments we present.

But even under the most favorable circumstances, it is evident that an absolute identity of experimental conditions is not to be attained. All that we can hope for is some degree of uniformity. Even in the same individual under these conditions there will be a range of variation in the ratio, a range which will express the extent of the failure to reproduce the same conditions during each repetition of the test.

Experiments defining the circumstances which influence the ratio will be given in a later paper. We show here the effect on the ratio of ligation of the ureter of one kidney in rabbits.

#### *The Ratio before and after the Ligation of One Ureter.*

This type of experimental lesion has been selected because it is one in regard to which our present methods of functional diagnosis fail. The functional changes they reveal are slight and entirely incommensurate with an anatomical alteration which involves the removal or destruction of half the renal tissue of the body. Thus it has been shown that when one kidney is removed,

there is no decrease in the amount or concentration of nitrogen or of urea in the urine, though there may be a moderate and temporary increase in the non-protein nitrogen of the blood.<sup>4</sup> Again, Rowntree and Geraghty<sup>5</sup> found when they tied the vessels of one kidney during a period when phenolsulfonephthalein was being excreted, that there was only a slight and not invariable decrease in the rate of excretion. Apparently the remaining kidney is able almost at once to carry on the work which was formerly done by both.

Our experiments were done on rabbits whose right ureters were tied after a segment had been excised. The ureter was reached through a lumbar incision without opening the peritoneum.<sup>6</sup>

The food throughout the whole period of observation, both before and after the operation, consisted of crushed oats. A weighed quantity, more than they needed, was given daily to each rabbit in a special receptacle designed to prevent the scattering of the food, and at the end of the 24 hour period the amount eaten was estimated by difference. Only 25 cc. of water were given at the commencement of each day. Since this was a smaller amount than they needed, all of it was drunk immediately after it was given.

For the first 7 days 24 hour urine collections were made. The amounts were completed by catheterization each day. On account of the low water intake, the quantities of urine were small and it was commonly found that no urine was passed in the cages, so that the whole 24 hour amount was obtained on catheterization. The frequently unavoidable loss incidental to the collection of urine in cages was thus avoided.

On the 8th day urine was collected by catheter over a period of 2 hours commencing about 1½ hours after the daily 25 cc. of water had been taken. A little more than 5 cc. of blood was taken from the ear vein at the middle of this period.

On the 9th day immediately after the water had been taken, 20 cc. of a 10 per cent solution of urea in 0.9 per cent NaCl were injected intraperitoneally, and 1½ hours later the collection of a 2 hour urine specimen was commenced and blood taken as before.

An interval of 10 days' freedom from observation was then interposed before the operation was performed. During this time a diet of green food, crushed oats, and unlimited water was given. This was similar to the diet taken by these animals before the experiment proper was started.

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<sup>4</sup> Karsner, H. T., Bunker, H. A., Jr., and Grabfield, G. P., *J. Exp. Med.*, 1915, xxii, 544.

<sup>5</sup> Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1912, ix, 284.

<sup>6</sup> Dr. Cowan kindly did this operation for us.

On the 20th day the right ureters were tied.

On the 21st day 24 hour collections of urine were made on the same diet as before operation. This was continued for 5 days.

On the 26th day a 2 hour specimen of urine and blood was collected, as had been done on the 8th day and again on the 27th day after the injection of the same amount of urea as had been given on the 9th day.

When the experiment was ended, the animals were killed. There was no trace of renal tissue on the right side. The capsule of the kidney was full of an amorphous cheesy material. Except for some thickening of the capsule, there was no indication of any inflammatory reaction in the surrounding tissues. There was no peritonitis. The remaining organs were normal. Dr. Jean Oliver examined the left kidneys microscopically and reported that there was no noteworthy departure from the normal.

The average data in regard to the 24 hour collections of urine for the 7 and 5 day periods before and after operation are given in Table I.

TABLE I.

*The Average 24 Hr. Urine and the Blood Urea Concentration before and after Ligature of the Right Ureter.*

	Rabbit 1.			Rabbit 2.			Rabbit 3.		
	Vol- ume.	Urea.	Blood urea per 100 cc.	Vol- ume.	Urea.	Blood urea per 100 cc.	Vol- ume.	Urea.	Blood urea per 100 cc.
	cc.	gm.		cc.	gm.		cc.	gm.	
Before ligature.....	28	1.12	0.043	45	1.16	0.099	41	1.47	0.046
After igature.....	52	2.38	0.082	64	1.85	0.143	47	1.82	0.056

The 24 hour amounts of urea excreted are increased after operation in all three rabbits. The increase is most marked in Rabbit 1, less in Rabbit 2, and least in Rabbit 3. Both the increase in urea and the variation in the degree of increase are the opposite of what one might have expected from the quantity of oats taken. For all three took less food after operation; Rabbit 1 ate only 5 per cent of its previous average daily amount, Rabbit 2, 10 per cent, and Rabbit 3, 67 per cent. There must therefore have been an increase in the rate of protein catabolism which was most marked in Rabbit 1, less in Rabbit 2, and least in Rabbit 3. It will be noted that the degree of increase in the blood urea concentration closely parallels the degree of this increase in protein



catabolism. This rise in the blood urea concentration after operation cannot be accepted as indicating a defective elimination of urea by the kidney, since we have found a similar parallelism between an acceleration of the rate of protein catabolism and a rise in the level of blood urea concentration in healthy individuals under physiological conditions.<sup>3</sup>

Table I shows that the kidney remaining after operation is able to excrete a larger amount of urea than was excreted by both kidneys together before the operation. It is also interesting to note that the concentration of urea in the urine is even higher after operation than before.

The ratios obtained before and after operation when no urea was given are shown in Table II.

TABLE II.

*The Ratio between the Urea in 1 Hr.'s Urine and the Urea in 100 Cc. of Blood before and after Ligature of the Right Ureter. No Urea Given.*

	Rabbit 1.			Rabbit 2.			Rabbit 3.		
	Urea in 1 hr.'s urine.	Urea in 100 cc. of blood.	Ratio.	Urea in 1 hr.'s urine.	Urea in 100 cc. of blood.	Ratio.	Urea in 1 hr.'s urine.	Urea in 100 cc. of blood.	Ratio.
	gm.	gm.		gm.	gm.		gm.	gm.	
Before ligation.....	0.0207	0.0432	0.46	0.0456	0.0990	0.46	0.0175	0.0456	0.38
After ligation.....	0.0425	0.0816	0.52	0.0819	0.1428	0.57	0.0455	0.0558	0.81

The ratios increase after operation.

As far then as the ratio may be taken as an index of function when no urea is given, it would appear that the single remaining kidney functionated more efficiently than both kidneys together before the operation, at least during the period and under the conditions present, when the observation was made.

The ratios before and after operation obtained after the injection of a 10 per cent urea solution are given in Table III.

The ratios decrease after operation.

Before the operation the urea injection had led to a considerable rise in the blood urea concentration, but there was a still greater increase in the amount of urea excreted, with the result

TABLE III.

*The Ratio between the Urea in 1 Hr.'s Urine and the Urea in 100 Cc. of Blood before and after Ligature of the Right Ureter. Urea Given.*

	Rabbit 1.			Rabbit 2.			Rabbit 3.		
	Urea in 1 hr.'s urine.	Urea in 100 cc. of blood.	Ratio.	Urea in 1 hr.'s urine.	Urea in 100 cc. of blood.	Ratio.	Urea in 1 hr.'s urine.	Urea in 100 cc. of blood.	Ratio.
	gm.	gm.		gm.	gm.		gm.	gm.	
Before liga- ture.....	0.1912	0.1560	1.23	0.2265	0.2790	0.81	0.3165	0.1728	1.83
After liga- ture.....	0.0682	0.1582	0.43	0.0682	0.3918	0.17	0.1850	0.1806	1.02

that the ratio was higher than in the experiment without urea. But after the operation, the remaining kidney did not respond in this way. The amount of urea excreted was less, and the blood urea concentration was higher, with the result that the ratio is lower than those given by both kidneys after urea. Under these conditions, therefore, the ratios indicate a defect in the urea-excreting function of the remaining kidney.

While we know that the ratio values even after urea injection cannot be regarded as mathematically accurate measurements of kidney function, we believe that the marked decrease after the removal of one kidney roughly represents the actual decrease in functional power, in spite of the fact that the opposite result was obtained after operation when no urea was given. The divergence arises from the difference in the conditions under which the two experiments were conducted, and both results may be substantially correct. Up to a certain point the kidney functions more efficiently under the strain of added work, but there is a point beyond which it cannot go. In this case the work the kidney was called on to perform is represented by the urea in the blood. The work it did is represented by the urea in the urine. Its functional capacity is represented by the ratio between the work done and the work to be done. In the first experiment, when no urea was given, the work to be done after the operation was moderately increased because of the greater rapidity of protein catabolism. The added strain was within the capacity of the remaining kidney, and it responded by a rise in

the ratio. In the second experiment when the work to be done was greatly increased by the injection of urea, the strain which before operation had stimulated both kidneys to greater efficiency, was too much for the remaining kidney; its maximal rate of work was less than the rate of increase in the work to be done, and there was a consequent decrease in the ratio.

This experiment illustrates an important principle in the measurement of kidney function. It shows that function may be entirely normal under ordinary conditions, and yet may show itself defective under strain. So the administration of a large quantity of urea before the ratio is determined has not only the advantage of tending to equalize the experimental conditions; it may also serve to bring latent deficiencies to light.

It is this type of test which may prove of value in the early diagnosis of disease of the kidneys in man. It is interesting in this connection to note that the rate of excretion of phenolsulfonaphthalein, which is acknowledged to be the best general test of kidney function, has shown itself to be of the greatest value in revealing the presence and to some extent the degree of pronounced disease of the kidneys, but has not proved efficient as a means for the discovery of the disease in its early stages. The strength of this latter type of test lies in the fact that the test substance is a foreign body not subject to any augmentation or decrease after injection into the body, and not held in the tissues, so that approximately the same proportion will reach the kidneys in all cases. Its weakness results from the excessively minute amount of the test substance which is injected, in the case of phenolsulfonaphthalein, for instance, only 0.006 gm. This does not impose any strain on the capacity of the kidneys. And since in the earlier stages of the progress of those pathological conditions which may be grouped under the term "chronic Bright's disease," the disease is, as a rule, not diffuse, but involves certain areas of the kidneys more than others and commonly leaves parts entirely unaffected, it comes about that the slightly or wholly unaffected sections of the kidney may find no difficulty in excreting this minute amount of dye in a manner entirely comparable to the mode of excretion of normal kidneys. A test of the type which we have described would seem to be better adapted for the diagnosis of such conditions.

## SUMMARY.

Urine and blood were collected simultaneously from rabbits, and the ratio

$$\frac{\text{Gm. of urea in 1 hr's. urine}}{\text{Gm. of urea in 100 cc. of blood}}$$

was determined before and after the destruction of one kidney by ligation of its ureter, while the animals were taking similar food and the same amount of water.

After the operation there was no decrease in this ratio as compared with the one found under similar conditions before operation, except when urea was injected.

The depression of the ratio after the operation when urea was injected is interpreted as indicating that the remaining kidney had less capacity for the excretion of urea than both kidneys before operation.

The fact that a depression of the ratio was found only when urea was injected is explained on the theory that the capacity of the remaining kidney was sufficient to meet ordinary demands, but showed itself defective under the strain of the additional work thrown on it by the urea injection.



## PANCREATIC DIABETES IN THE DOG.

### IV. THE INFLUENCE OF PYLORUS EXCLUSION AND OF GASTRECTOMY UPON THE EFFECTS OF PANCREATECTOMY.

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#### INTRODUCTION.

That an intimate relationship exists between the acid-producing function of the stomach and the alkali-producing function of the pancreas has long been known. Pawlow and his pupils (1) have demonstrated that the amount of alkaline ash present in the pancreatic juice depends upon the strength of acid used as an exciting agent. Cohnheim and Klee (2) have furnished proof that the amount of pancreatic juice produced by any food is proportional to the amount of gastric juice which that food calls forth, and Wilbrand (3), in Cohnheim's laboratory, measuring the outflow of pancreatic juice following the administration of sodium bicarbonate by stomach, found that it is reduced in amount in reverse fashion to the effect of acid. Worobjeff (4) found that strong alkali injected intravenously acted in the same way.

The importance of this regulation through the secretin mechanism for events within the alimentary canal is readily understood. New evidence of its importance has come to light through the work of Boldyreff (5) and his pupils on the self-regulation of the acidity of the stomach contents, and through the work of Rehfuess and Hawk (6) on the stimulating action of alkali, among other agents, on the gastric glands.

As far as we can discover, the importance of this regulation of intestinal alkalinity to stomach acidity for events beyond the alimentary wall, has not been sufficiently considered. If, as the work cited above seems to prove, it is essential to health that gastric acidity be completely neutralized, what would be the consequence of preventing such neutralization by removal of the pancreas, considering that the pancreas, as Boldyreff shows, produces more than ten times as much alkali as appears in the bile and twenty times as much as in the intestinal juice? The effects on digestion of removal of the pancreas are sufficiently known (7) and are serious enough; for only 44 to 70 per cent of the protein ingested can be pre-

pared for absorption by the peptic digestion alone, while about 33 per cent, at best, of the fat (8) can be utilized and none of the carbohydrate. So far the change represents merely lack of function, not derangement. But what is the consequence of permitting free hydrochloric acid to remain unneutralized in the intestine?<sup>1</sup>

Elias (9) produced glycosuria by feeding animals with hydrochloric acid, and perfused livers with a fluid to which hydrochloric acid had been added, and proved that glycogen rapidly disappeared therefrom. Elias and Kolb (10) demonstrated that the hunger diabetes of Hofmeister is due to an acidosis and that it could be prevented by injection of alkalies. Going further back, we find that Naunyn in 1868 (11) had observed glycosuria in a dog poisoned with hydrochloric acid, and Külz (12) had considered the subject at some length in 1881. A greatly reduced protection against the toxic action of hydrochloric acid by the dog after pancreatectomy has been demonstrated by Eppinger (13) and has been confirmed by Murlin and Kramer (14) in the first paper of this series. The work of Schryver (15) makes it probable that the loss of the power to produce ammonia from protein following tryptic digestion is, in part, the explanation of this reduced tolerance.

There are, however, still further consequences of acid in the circulation. Feeding rabbits with foods which yield an acid ash produces, as Underhill (16) has shown, creatine in the urine, a sign of abnormal catabolism in the muscles, and acid favors autolytic changes in the tissues (17). As is well known also, it reduces the carbon dioxide tension. It may even increase the heat production, quite aside from the effects of dyspnea.

Several of the consequences of acid intoxication are, therefore, coincident with (if not identical with) the consequences of pancreatectomy; namely, glycogenolysis and glycosuria, autolysis (18), and increased heat production. This coincidence, when it was first realized some 3 years ago, suggested to one of us (19) the possibility that the consequences of pancreatectomy might be due in large measure to an acid intoxication resulting from loss of sufficient alkali-producing function.

There are many supporting and contributory facts, some of which should be mentioned at this point. For example, Minkowski (20) is authority for the statement that only carnivorous animals (including carnivorous birds) that are known to have a gastric juice of higher acidity than that of herbivorous animals get a severe diabetes after pancreatectomy. It is well known, also, that a meat diet gives the most severe toxemia with dogs after the pancreas is removed, and it is equally well known that a meat diet stimulates gastric secretion more than any other. Meat tends to break down the tolerance of partially depancreatized dogs,

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<sup>1</sup> According to a personal communication from A. J. Carlson, the secretion of gastric juice is greatly augmented after pancreatectomy. We have received this impression also from a single case of pancreatectomy following a Pawlow operation on the stomach. A quantitative comparison, however, was not possible.

as we have seen in the previous papers, and aggravates human diabetes,<sup>2</sup> while, as Allen (21) states, a long continued excess of sugar does not appear to predispose to diabetes. Again, the favorable effects of starvation, both on human and experimental diabetes, now well known through Allen's work, may be explained as due in part at least to a reduction of gastric activity. The processes of metabolism beyond the alimentary wall are believed to be essentially the same in quality in fasting as in alimentation; hence the favorable effect of fasting, aside from the mere reduction or exhaustion of the glycogen supply (which is not the source of the sugar in severe diabetes), must be due to the abatement of some exciting cause originating in the alimentary organs themselves. The favorable effects of green vegetables, which for the most part have an alkaline ash (22), and of oatmeal, which, while having an acid ash, has, according to Cohnheim and Klee (2), but a feeble secretogenic effect on the gastric glands, are in line with the explanation of fasting. The ligation of the pancreatic ducts has been reported by Allen<sup>3</sup> and by Homans (23) to have a favorable effect on the course of the diabetes in the partially depancreatized dog. While this may be explained in part by the diminished digestion of starchy materials (and of meat, for that matter), it may be supposed also that alkali, separated from its salt or protein combination in the blood by the pancreas, can be poured back into the circulation when the ducts are blocked and may be secreted by the intestinal wall or carried as such to the liver and in this way insure the protection of that organ against the toxic effects of hydrochloric acid. It must be remembered, however, that Wohlgemuth (24), by ligation of the pancreatic ducts in normal dogs, has produced a hyperglycemia and a diminished power to oxidize glucose (low respiratory quotient). The same effects may be produced by injection of hydrochloric acid.

The experiments of Macleod and Pearce (25), confirmed by Patterson and Starling (26), showing that animals eviscerated after pancreatectomy have as great a capacity to remove glucose from the circulation as have animals previously normal, may be interpreted as meaning that some alimentary organ produces a toxin<sup>4</sup> which normally is counteracted or removed by the pancreas. The fact that such animals after pancreatectomy and subsequent evisceration cannot oxidize glucose within the short time that they survive, as Verzář (27) has shown, may mean only that irreparable injury had already been done before evisceration.

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<sup>2</sup> See the case of Joseph D. in a forthcoming paper by Gephart, Aub, and Du Bois in which it is shown that a man whose urine had been rendered sugar-free by a reduced diet was suddenly brought to a G:N ratio of 3.6 by a large meal of meat.

<sup>3</sup> Allen (21), page 1045.

<sup>4</sup> The conviction that the stomach contributes in some way to the etiology of diabetes mellitus has been expressed by a number of clinical writers, among whom may be mentioned Sawyer of Cleveland, Dietrich of Petrograd, and Funck of Berlin.



The work presented in the previous papers of this series has sufficiently shown, we believe, the importance of alkali for the combustion of glucose. Just how this alkali acts, whether by influencing the chemical reaction or by affecting the protoplasmic mechanism, is not yet clear, although the importance of an exact regulation of the hydrogen ion concentration for the processes of glycolysis and oxidation is indicated by the work of Rona and Wilenko (28), who have shown that a slight change in hydrogen ion concentration greatly modifies the consumption of sugar in the rabbit heart, and by Rona and Neukirch (29), who show that the utilization of sugar by intestinal loops is much better in Tyrode's solution ( $C_H = 0.2 \times 10^{-7}$ ) than in Locke's solution ( $C_H = 0.2 \times 10^{-6}$ ). If the utilization of glucose by the excised heart or gut is sensitive to an absolute change as slight as this, does it not appear probable that the exact neutralization of hydrochloric acid of the stomach in the living animal is of even greater moment for the carbohydrate metabolism of the liver and other tissues?

The anatomical relationships of the portal circulation take on new physiological significance with this conception. When acid is taken from the blood by the gastric glands the venous blood in the gastric veins must be relatively richer in hydroxyl ions. Accepting the view commonly held that the chlorine ions of hydrochloric acid are derived from sodium chloride in the blood, there would then be a relative excess of sodium ions left behind. Normally, this disturbance as regards sodium is at once restored by the production of the sodium carbonate by the pancreas (1), for not only is the amount of alkali produced by the pancreas proportional to the production of acid by the stomach, but, as appears clearly in the work of Pawlow's pupils, its secretion begins *as soon as the production of acid begins in the stomach*. The blood from gastric and pancreatic veins, reunited in the portal, has then a normal reaction before it enters the liver. In some instances it would seem that the pancreas does not keep pace with the stomach, for alkaline urines have been reported early in gastric digestion. When the pancreas is removed entirely we might expect also that the kidney would remove the excess of sodium ions resulting from secretion of hydrochloric acid into the stomach until this acid were again absorbed into the blood. *Even if this*

were not the case, however, the liver would not suffer from a temporary excess of such ions (combined probably as disodium hydrogen phosphate or as sodium carbonate or with protein), for the tolerance of protoplasm for free hydroxyl ions is much greater than for free hydrogen ions. The trouble would come when the hydrochloric acid reached the duodenum.

The secretin mechanism is proof of the permeability of the duodenal wall to this acid, and the mechanism which Boldyreff has discovered by which duodenal contents are regularly regurgitated into the stomach of the dog, whereby the acidity of the chyme is kept down to about 0.15 per cent hydrochloric acid, is proof of the importance of some protection against higher concentrations in the duodenum. This was proved directly also in Boldyreff's experiments by administration of higher concentrations. "With a stronger acid pathological symptoms at once appear. Undiluted stomach contents produce a bloody secretion usually followed by vomiting. The animal becomes very restless."<sup>5</sup> We shall cite an instance in confirmation of these statements.<sup>6</sup>

Pawlow<sup>7</sup> likewise has noted incidentally that dogs with permanent pancreatic fistulas could be kept in health only by the administration of alkali and by excluding meat (because of its exciting effect on the gastric glands) from the diet. It would be interesting to know whether these dogs would show a diminished tolerance for sugar.

Sufficient evidence has already been cited above and in the first paper of this series of the extreme sensitiveness of the liver to any excess of acid. As Mathews (30) puts it: "In the liver every acidosis is at once accompanied by the transformation of some glycogen into glucose," as a protection against its effect upon the processes of oxidation. Our conception is that in the depancreatized dog the hydrochloric acid of the gastric juice cannot be completely neutralized by the alkali of the bile and intestinal juices; it is therefore absorbed<sup>8</sup> in sufficient amount

<sup>5</sup> Boldyreff (5), page 156.

<sup>6</sup> Page 278.

<sup>7</sup> Pawlow (1), page 8.

<sup>8</sup> Obviously the HCl would not exist as such in the blood, and it is quite possible that the toxic effect is due to some acid-protein combination formed either in the alimentary wall after the manner of secretin, or formed in the blood itself.

by the portal circulation to injure the liver. Glycogen is thus liberated, oxidation is interfered with, more acids (acetone bodies) are formed, and a vicious circle is thus established. We shall show that this accounts for the rapid onset, at least, of diabetes after pancreatectomy.

#### EXPERIMENTAL.

Several ways of testing this hypothesis suggested themselves. By ligation of the pylorus at the time of pancreatectomy the acid chyme could be prevented from entering the intestine. Secondly, the stomach could be completely removed, preferably before the pancreas was removed, or simultaneously with it. The consequences of pancreatectomy in either case should be quite different from those following simple pancreatectomy. All of these methods have now been tried.

In July, 1913, Kramer and Murlin ligated the pylorus at the time the pancreas was removed in three different dogs. The details are given in Experiment 1. When the pancreas is removed from a dog in good nutritive condition sugar appears in the urine within a very few hours. Underhill and Fine (31) found as much as 7.3 gm. of glucose in the urine passed 2 hours after pancreatectomy. In the series of dogs depancreatized in the Cornell Laboratory within the past 4 years, unless the animal was subjected to some special treatment, sugar has been found invariably in the cage urine of the subsequent night. In many instances taken at random through the series it has been found in abundance within 4 hours, though no quantitative estimations have been made as early as this.

#### *Experiment 1. Effect of Ligating Pylorus.*

*Dog I.*—Brown spaniel, female; weight 15.0 kg.; in good nutritive condition.

July 25, 1913. Pancreas removed and stomach ligated off by pure gum tubing tied firmly about the duodenum just above the bile duct. No interference with blood vessels outside portion compressed. Stomach washed out with water made alkaline with sodium carbonate. Finished at 5 p.m. 500 cc. of Ringer's solution subcutaneously.

July 26, 9.30 a.m. Urine by catheter sugar-free, acid; temperature 39.4°. 500 cc. of Ringer's solution subcutaneously. 5.30 p.m. Urine by catheter sugar-free, acid; temperature 39.8°. Immediately after this the

stomach was washed with a weak solution of sodium carbonate. Dog vomited, breaking large blood vessel; died at once. Abdomen found full of blood. Duodenum perfectly healthy; ligature competent; no sign of peritonitis.

*Dog II.*—Boston bull terrier; weight 10 kg.; in good condition.

July 28. Pancreas removed complete and duodenum ligated with pure gum tubing just above bile duct. Completed at 4.30 p.m. Stomach washed out with sodium carbonate immediately after operation. Warm Ringer's solution subcutaneously.

July 29, 4.30 p.m. Urine from cage negative to Benedict's solution; temperature 38.2°. 500 cc. of Ringer's solution subcutaneously. Stomach not washed. Dog apparently in good condition.

July 30. Dog found dead. Urine from cage gave very faint reaction for glucose. Autopsy revealed general peritonitis, probably originating from the fistulous opening made by the rubber tubing, the end of which was secured to the under surface of skin. Duodenum entirely smooth; reduced to size of lead pencil.

*Dog III.*—Mongrel, female; weight 6 kg.; in good nutritive condition.

July 31. Pancreas removed and duodenum ligated above bile duct, leaving rubber tube inside. Completed at 1 p.m. Stomach washed out with alkali before operation. 5 p.m. 400 cc. of Ringer's solution subcutaneously. Temperature 39.4°.

Aug. 1, 10 a.m. Urine by catheter contains sugar. G:N = 0.88. Temperature 39.4°. 1.45 p.m. 600 cc. of Ringer's solution subcutaneously.

Aug. 2, 12.30 p.m. Urine by catheter sugar-free; temperature 40.4°. 1 p.m. 500 cc. of Ringer's solution subcutaneously. 1.10 p.m. Placed in ice box (temperature 11–14°). Out at 2.40 p.m. Urine by catheter sugar-free; temperature 39.8°. Superficial wound cleaned, evacuating 15 to 20 cc. sanguinopurulent fluid. Washed with alcohol and tincture of iodine. Returned to ice box. 5.30 p.m. Urine by catheter sugar-free; temperature 39.2°. 500 cc. of Ringer's solution subcutaneously.

Aug. 3. Dog found dead.

*Autopsy.*—No pancreatic tissue. Rubber ligature competent, but duodenum perforated just above; large localized abscess.

In these dogs with the pylorus ligated, however, sugar failed to appear within the first 24 hours in two out of the three cases, and in the third was present in very small amount. Two of the three dogs died by accident; *i.e.*, infection was not the primary cause, while the third died of a generalized peritonitis. We have seen many dogs with peritonitis, however, that excreted large quantities of sugar. Since in most cases the first 24 hour urine of a well nourished dog after simple pancreatectomy exhibits a high G:N ratio—often as high as 6 and usually 4 or 5—the results shown by these first animals seemed positive

enough to follow them up with a series of gastrectomies. Excluding the acid chyme from the intestine at the time of operation appeared to prevent or at least greatly retard the onset of glycosuria. Complete removal of the stomach previous to pancreatectomy should be a still more crucial test.

Dr. John A. Hartwell became interested in the problem from the surgical point of view and successfully removed the stomach and pancreas from three dogs. One of these died from infection and will be omitted from the report, although the result was similar to that obtained with Dog V except that sugar appeared in small amount (G:N ratio 1.09) on the morning following the operation.

Both dogs reported in Experiment 2 died accidentally, one on the 3rd day after pancreatectomy by rupture of the abdominal wound, the other on the 4th day by rupture of the inverted end of the esophagus. The onset of diabetes, therefore, was not checked or modified in any way by infection.

Neither of these dogs developed any glycosuria on the day following pancreatectomy. Both developed it later, but in both cases the G:N ratio was very low, so that one could say that the dog presented a very mild type of the disease.

*Experiment 2. Gastrectomy before and Simultaneously with Pancreatectomy.*

*Dog IV.*—White mongrel, male; weight 13 kg.

Oct. 21, 1913. Dr. Hartwell removed the stomach; gastro-enterostomy between cardia and intestinal wall well below pancreas. Blind end of duodenum inverted just above bile duct and anchored to peritoneum. About 2 inches of gastric mucosa left below cardia.

Oct. 22-24. Dog fed only fluid foods.

Oct. 24-27. Fed small amounts of meat; condition good.

Oct. 27, a.m. Pancreas removed complete. Everything in good condition except that a small abscess was found about the blind end of the duodenum.

Oct. 28. Urine sugar-free.

Oct. 29. Urine from cage (colored with bile) gave strong reaction for sugar. G:N = 1.11. 12 m. Temperature 39°. Few drops of urine positive for sugar. 3 p.m. Cage urine gives strong Pettenkofer's test for bile. Dog drank 500 cc. of Ringer's solution, 300 cc. of it containing 1 per cent sodium bicarbonate.

Oct. 30. Morning urine from cage. G:N = 1.08. P.m. Dog found with wound open; killed. Rupture probably due to vomiting. Had not been able to tolerate food since last operation.

*Dog V.*—Brown bull; female; ears cut; weight 14 kg.; in splendid nutritive condition.

Nov. 12, 1913, 2-4 p.m. Dr. Hartwell removed the pancreas and stomach entire. No anastomosis. Esophagus inverted; duodenum inverted.

Nov. 13, 10 a.m. Urine by catheter negative to Benedict's solution; temperature 38°. 400 cc. of sterile Ringer's solution subcutaneously. 6 p.m. Urine by catheter negative to Benedict's solution; temperature 38.4°. 500 cc. of sterile Ringer's solution subcutaneously.

Nov. 14, 10 a.m. Urine by catheter positive to Benedict's solution. G: N = 0.38, temperature 38.4°. 500 cc. of Ringer's solution subcutaneously.

Nov. 15, 4 p.m. Urine by catheter, G: N = 0.94. 750 cc. of Ringer's solution subcutaneously.

Nov. 16, 12 m. Urine by catheter, G: N = 1.04; temperature 38.6°. 12.30-1 p.m. Infusion of 150 cc. of Ringer's solution. Dog ruptured esophagus and swallowed air into peritoneum while on table. Air let out by puncture through abdominal incision. Dog died during night.

*Autopsy.*—No pancreatic tissue.

Nothing more was done on the problem until June 1, 1914, when one of us (Murlin) operated on a dog in a different manner. Instead of ligating the pylorus, the same result was obtained by complete occlusion; the intestine was severed just below the pylorus and the two cut ends were inverted. The pancreas was removed entire.

### *Experiment 3.*

*Dog VI.*—Female; weight 10 kg.

June 1. The operation was done under morphine and ether and the dog was given 2 cc. of 2 per cent morphine after the operation.

June 2. Urine from the cage gave no reaction for sugar and at 4 p.m. urine from the bladder was still negative. The dog's temperature, however, was only 36°, possibly due to the heavy morphine sleep of the night before. 300 cc. of sterile water were given subcutaneously.

On June 3 the dog was found to be in good condition, the urine from the cage showing only a faint trace of sugar. The temperature was 38.8°. At 4 p.m. urine from the bladder contained no sugar. During the day the dog had vomited some bloody detritus from the stomach and for fear of infection the stomach was washed with boric acid at about 4.30 p.m. Up to this time the dog had been in good condition, active and strong. After the treatment with boric acid it collapsed and died about 6 o'clock.

*Autopsy.*—No adequate cause of death evident. No pancreatic tissue could be seen.

These results seemed to warrant a more detailed study. Dr. Sweet<sup>9</sup> successfully operated four dogs which survived without any complications and have been studied exhaustively.

The first of these, Dog VII, was operated on June 5, 1914. The entire stomach and a major portion of the pancreas were removed at this time. 11 days later the remainder of the pancreas was taken. Meantime the dog had eaten meat every day.

The urines were taken from the cage every morning for the next 17 days and analyzed for nitrogen and glucose.<sup>10</sup>

TABLE I.

*Dog VII. Effect of Gastrectomy on Pancreatic Diabetes Produced Subsequently.*

June 5, Stomach removed with major part of pancreas.

" 16, 11 a.m. Remainder of pancreas removed.

" 17, 6.30 a.m. No sugar in urine.

" 17, 6 p.m. No sugar in urine.

Date.	Weight.	Glucose.	Nitrogen.	G : N.
	kg.			
June 18.....		3.96	4.35	0.90
" 19.....		10.41	6.03	1.7
" 20.....	11.81	7.97	7.5	0.94
" 21.....	11.40	5.04	5.8	1.15
" 22.....	11.36	4.59	4.2	1.09
" 23.....	11.00	5.0	6.45	0.77
" 24.....	10.65	6.25	5.34	1.17
" 25.....	10.39	7.9	5.51	1.25
" 26.....	10.12	3.75	5.34	0.70
" 27.....	9.95	3.54	3.62	0.98
" 28.....	9.80	3.96	2.75	1.44
" 29.....	9.76	3.96	2.70	1.47
" 30.....	9.45	3.33	2.73	1.22
July 1.....	9.30	3.33	2.92	1.14
" 2.....	9.00	4.20	2.92	1.44
Average.....				1.17

July 2. Animal killed with chloroform.

*Autopsy.*—Anastomosis complete; no pancreatic tissue.

<sup>9</sup> Dr. Sweet took charge of the surgical work from June, 1914.

<sup>10</sup> The analytical work was done in the laboratory of Professor A. E. Taylor by Dr. H. B. Lewis. We wish to express our thanks for their cooperation.

After complete pancreatectomy the dog had no sugar in the urine for the first 36 hours or more. He then developed the same mild type of diabetes seen in Dogs IV and V, and this persisted without much variation until July 2, the 17th day after pancreatectomy, when the dog was killed.

Another point of particular interest was the absence of the severe toxemia which one ordinarily sees after pancreatectomy. The dog emaciated, as is usual after the simple operation, but did not appear to be sick. He always ate greedily but the food (meat) came through entirely undigested. So striking was the difference between this animal and the victim of simple pancreatectomy that his survival for so long a time was a matter of remark. The case gave a distinct impression that the chemical processes of alimentation in some way contribute to the severe toxemia of pancreatic diabetes. The physical processes, chewing, swallowing, peristalsis, were all present. The dog probably would have lived at least a week or 10 days longer.

The remaining dogs to be considered have been operated within the past year in the Cornell Laboratory by Dr. Sweet. In each case, in the first operation a gastrectomy was done as completely as possible but the pancreas was not molested. The object of this was to gain some knowledge of the utilization of food in the absence of gastric digestion and to determine whether the state of nutrition might in any way affect the results after pancreatectomy.

Minkowski and others have shown that unless a dog is in good nutritive condition at the time of pancreatectomy the resulting glycosuria may be very mild. For this reason every effort was made to maintain proper alimentation after gastrectomy. When Dog VIII continued to lose weight on what seemed to be a sufficient amount of food for a normal dog an imitation of gastric digestion was restored by giving 0.3 per cent hydrochloric acid and lactopeptine which contains an active pepsin. With this diet she maintained her weight for 8 or 9 days and when the acid and digestive powder were discontinued the appetite appeared to fail at once. 2 days later she refused food. Meat alone was then tried and was taken readily. On the 7th and 8th a mixed diet was offered once more and artificial digestion was once more resorted to. The dog made a distinct gain and apparently could be said to have recovered a satisfactory state of nutrition when the pancreas was removed.



TABLE II.

*Observations after Gastrectomy. Dog VIII, Young White Female, Partly Bull; Weight 9.5 Kg. Gastrectomy November 11, 1915, 9.50 to 11 a.m. Anastomosis Well below the Pancreas.*

Date.	Temperature. °C.	Weight. kg.	No. of feedings.	Food at each feeding.	Remarks.
Nov. 12....	38.8			Drank a little milk.	No vomiting.
" 13....	38.8	8.35	2	100 gm. meat.	" "
" 14....	38.6		1	300 " "	
" 15....	38.1	8.10	3	Small amount of meat and lard.	
" 16....	38.8	8.20	2	100 gm. meat; 30 " lard; bone ash.	
" 17....	38.2	8.10	2	Same.	
" 18....	38.2	8.00	1	100 gm. meat; 30 " cracker meal; 10 " lard; bone ash.	
" 19....		7.95	2	Same, except 20 gm. lard.	
" 20....	37.8	8.00	1	100 gm. meat; 20 " lard; 30 " cracker meal; bone ash.	
" 21....			2	Same.	
" 22....		7.85	2	"	
" 23....		7.75	2	Same food + 0.3 per cent HCl + lacto- peptine.*	
" 24-25.			2	Same.	
" 26-28.		7.80	2	"	
" 29....		7.80	2	"	
" 30....			2	"	
Dec. 1....		7.80	2	Same food but no HCl and no lactopeptine.	
" 2....		7.80	1	Same.	Did not eat well.
" 3....		7.70	1	"	" " " "
" 4....		7.60			Refused food.
" 5....		7.45	2	200 gm. meat.	All eaten.
" 6....		7.35	2	200 " " 50 " cane sugar.	
" 7....		7.40	1	200 gm. meat; 30 " cracker meal; 10 " lard; 1 pt. milk; lactopeptine.	All eaten.
" 8....		7.45	1	Same.	Not quite all eaten.
" 9....		7.50		No food.	Pancreas extirpated.

\* New York Pharmacal Association. See note 16.

The pancreas was found to be normal in every respect except that the peritoneum was thickened about the uncinat process. There were no troublesome adhesions. The operation was completed in 45 minutes. With this dog we began the study of the respiratory metabolism.

TABLE III.

*Dog VIII. Gastrectomy November 11. Pancreatectomy December 9, 1915.*

Date.	Time.	Weight.	Urine.			Respiratory metabolism.			Food and remarks.
			Total glucose.	Total nitrogen.	G : N.	CO <sub>2</sub> per hr.	O <sub>2</sub> per hr.	R. Q.	
		kg.	gm.	gm.		liters	liters		
Dec. 10	9.10	7.05	2.50	2.16	1.11				Given milk by mistake.
" 11	9.35	6.90	0.305	0.496	0.65				
	10.17-11.17					2.434	3.453	0.73	50 gm. glucose by tube, vomited. 20 gm. glucose subcutaneously.
	11.17-12.17					2.511	3.331	0.75	
	12.35		0.244	0.40	0.61				
	3.45- 4.46					3.120	4.030	0.77	
	5.15- 6.15					3.039	4.000	0.76	
	6.15- 7.15					3.149	4.316	0.73	200 cc. milk.
" 12									
" 13	3.42- 4.42	6.05				2.817	3.921	0.72	No food.
	4.42- 5.42					3.596	4.948	0.73	
" 14	11.15	6.05	0.0						8.00 p.m. fed 30 gm. starch and lactopeptine.
" 15	4.35	6.05	0.0						
	4.58- 5.58					2.413	3.408	0.71	
	5.58- 6.58					2.465	3.427	0.72	
	7.35		0.0	0.378					
" 16	a.m.	6.15	2.84						1.10 p.m. fed 50 gm. cracker meal with dog's pancreas.
	12.20		0.0						
	3.25- 4.25					3.654	4.994	0.73	
	4.25- 5.25					2.873	3.998	0.72	

On December 10 the dog excreted some sugar but exhibited a G : N ratio of only 1.1, instead of a ratio in the neighborhood of 4 or higher which well fed dogs usually exhibit on the 1st day (Table III). In spite

TABLE II.

Observations after Gastrectomy. Dog VIII, Young White Female, Partly Bull; Weight 9.5 Kg. Gastrectomy November 11, 1915, 9.30 to 11 a.m.  
Anastomosis Well below the Pancreas.

Date.	Temperature.	Weight.	No. of feedings.	Food at each feeding.	Remarks.
	°C.	kg.			
Nov. 12....	38.8			Drank a little milk.	No vomiting.
" 13....	38.8	8.35	2	100 gm. meat.	" "
" 14....	38.6		1	300 " "	
" 15....	38.1	8.10	3	Small amount of meat and lard.	
" 16....	38.8	8.20	2	100 gm. meat; 30 " lard; bone ash.	
" 17....	38.2	8.10	2	Same.	
" 18....	38.2	8.00	1	100 gm. meat; 30 " cracker meal; 10 " lard; bone ash.	
" 19....		7.95	2	Same, except 20 gm. lard.	
" 20....	37.8	8.00	1	100 gm. meat; 20 " lard; 30 " cracker meal; bone ash.	
" 21..			2	Same.	
" 22....		7.85	2	"	
" 23....		7.75	2	Same food + 0.3 per cent HCl + lactopeptine.*	
" 24-25.			2	Same.	
" 26-28.		7.80	2	"	
" 29...		7.80	2	"	
" 30....			2	"	
Dec. 1....		7.80	2	Same food but no HCl and no lactopeptine.	
" 2....		7.80	1	Same.	Did not eat well.
" 3....		7.70	1	"	" " " "
" 4....		7.60			Refused food.
" 5....		7.45	2	200 gm. meat.	All eaten.
" 6....		7.35	2	200 " " 50 " cane sugar.	
" 7....		7.40	1	200 gm. meat; 30 " cracker meal; 10 " lard; 1 pt. milk; lactopeptine.	All eaten.
" 8....		7.45	1	Same.	Not quite all eaten.
" 9....		7.50		No food.	Pancreas extirpated.

\* New York Pharmacal Association. See note 16.

The dog was killed about 10 days later, but probably would have lived at least a week longer. The same absence of severe toxemia was evident in this dog as in Dog VII. The wound healed promptly after pancreatectomy and the animal was always playful and alert even when she had become much emaciated. No pancreatic tissue was found at autopsy.

We were fortunate in having two white bull terrier females of about the same weight and age. One of these, Dog XV, was gastrectomized on January 3, and the other, Dog XVI, on January 7. The former was a ravenous eater and offered, therefore, an unusual opportunity to study the nutrition of a dog after a total, or nearly total,<sup>13</sup> gastrectomy. Dog XVI was not a good eater and all attempts to secure quantitative results on the utilization of food were frustrated.

### *Nutrition of the Dog after Gastrectomy.*

The most recent work on the nutrition of the dog after gastrectomy was done by Levene and his coworkers (32) who showed that after both gastro-enterostomy and gastrectomy the retention of extra nitrogen superimposed on a standard diet was greatly interfered with. The explanation, according to these authors, is that the stomach normally prepares protein food for assimilation by only partially digesting it, while the intestine carries digestion to its end stages in order to facilitate its further disposition toward excretion. The stomach being emptied too soon, as after gastro-enterostomy, or being entirely removed, digestion goes on too rapidly for retention. Their animals had no difficulty, however, in maintaining nitrogen equilibrium.

The fact last stated is an important one for our problem because it made it almost certain that Dog VIII was in nitrogen equilibrium as well as in energy balance (as judged by weight) at the time of pancreatectomy. Nevertheless, in order to be secure on this point, it was necessary to study both the nitrogen balance and the fat balance. Accordingly, a diet was selected for Dog XV which should certainly contain sufficient nutrients of all classes, and the feces were carefully separated in periods,

accomplished through a different agency than the regulation of the reaction of the blood. Compare the views of Rona and Wilenko (28). We are indebted to Professor Lusk for calling our attention to the hypothesis of Rona and Wilenko, which is so nearly akin to the one which prompted this research.

<sup>13</sup> It was necessary to leave a ring of gastric wall about 2 cm. wide below the cardia in order to make a secure anastomosis with the intestine.

TABLE IV.  
Dog XV. Nutrition without Stomach.

Period.	Date.	No. of days.	Weight average for period.	Food.	N in food.	Calories in food per kilo.	N in urine, average per day.	N in feces, average per day.	Utilization of protein, per cent.	Total N.	N balance, gm.	Fat in food.	Fat in feces per day.	Utilization of fat, per cent.	Fat balance, gm.
I	Jan. 11-13	3	10.90	200 gm. beef heart; 30 " lard; 300 cc. milk; 50 gm. cracker meal; 10 " bone ash.	7.99	71	5.51	1.66	77.0	7.17	+0.82	50.6	8.38	83.0	+42.2
II	" 14-17	4	10.88	Same only 250 cc. milk + 0.25 per cent HCl.	7.75	69	4.87	1.09	86.0	5.96	+1.79	49.1	8.41	83.0	+40.7
III	" 18-20	3	10.70	Same + 2 gm. scale pepsin + acid.	8.01	71	5.44	1.18	83.3	6.62	+1.39	49.1	8.33	83.0	+40.8
IV	" 30-31	2	10.25	400 gm. beef heart; 30 " lard; 10 " bone ash.	11.36	69	7.18	2.43	77.0	9.61	+1.75	46.5	8.96	80.8	+37.5
V	Feb. 1-5	5	10.23	Same + 10 gm. lactopeptine No. 2617.	11.73	74	8.29	1.95	83.4	10.24	+1.49	46.7	6.44	86.2	+40.3

VI Feb. 6-7 2 10.15 400 gm. beef heart.

VI	Feb. 6-7	2	10.15	400 gm. beef heart; 30 " lard; 50 " cracker meal; 10 " bone ash.	12.23	93	8.75	1.27	89.6	10.02	+2.21	49.8	8.09	84.8	+41.7	
VII	"	8-12	5	10.37	Same + 10 gm. lac- toeptide No. 2639.	12.58	94	8.83	1.14	90.9	9.98	+2.60	50.1	5.05	90.0	+45.1
VIII	"	13-14	2	10.30	Same without lac- toeptide.	12.23	90	8.84	1.68	86.3	10.52	+1.71	49.8	8.29	83.4	+41.5

part of the time by means of carmine and part of the time with silicic acid. Dogs of the same breed have been kept in good nutritive condition for long periods of time in the Cornell Laboratory by a diet of the same general character. Small and frequent meals were given at first in order not to put too great a strain upon the anastomosis, but the dog soon developed a capacity for a larger amount and at the end of the 1st week was taking the 24 hour diet in two feedings without any apparent discomfort. This same number of feedings was continued throughout the experiment.

With 7.99 gm. of nitrogen and 71 calories per kg. of potential energy in the food during the first 3 day period, the dog retained 0.82 gm. of nitrogen daily and utilized 77 per cent of the protein intake and 83 per cent of the fat (Table IV).

With the idea that this utilization might be improved by stimulation of the pancreas through formation of secretin, hydrochloric acid was administered by a tube introduced into the duodenum. For the first dose 0.5 per cent acid was used, but this produced violent peristalsis and bloody stool, which continued for some hours. The dog drank water freely, but it was largely vomited. This observation confirms the statements of Boldyreff<sup>14</sup> in every particular. The strength of acid subsequently was reduced to 0.25 per cent and it was fairly well borne, although it was noticed that the dog drank more water than usual.

The utilization of food in this period was determined by careful separation of the well formed stool from blood which followed the first administration of acid. The protein had been absorbed to a greater extent, but the fat in the stool was not changed, showing that such benefit as was derived from the acid stimulation accrued through assistance to the proteolytic rather than to the lipolytic digestion. The query is naturally suggested whether this is an instance of the specificity of secretion insisted upon by Walther (33) and others whereby, in this instance, acid called out by protein in the stomach may, in turn, call out especially the enzyme for its digestion.

Scale pepsin<sup>15</sup> was next given for a period of 3 days together with the acid, but the net utilization of food was not changed.

<sup>14</sup> Page 265.

<sup>15</sup> Parke, Davis and Company.

This does not mean that digestion was not improved a little, for the amount of protein ingested was somewhat greater than in the previous period, and the amount of nitrogen in the urine was greater.

At this point the dog became ill of distemper (skin eruption, fever, and a cough) so that the experiment had to be discontinued. Fortunately, the trouble was of short duration. The fever completely disappeared within a week and the appetite remained as vigorous as ever. Since the dog had lost weight through the first three periods the diet was changed so as to furnish more protein, and 10 days later to furnish more potential energy as well. The higher protein was continued for 3 days before the scientific observations were resumed. Because the first stools were soft, a large amount of bone ash was given.

The utilization of protein in the first period of observation was exactly the same as in the first period on the lower protein but the utilization of fat was not quite so good. With the help of lactopeptine<sup>16</sup> the utilization of both protein and fat was considerably improved, that for protein increasing from 77 to 83 per cent,<sup>17</sup> and for fat from 81 to 86 per cent. When cracker meal was added to the diet the digestion of protein for the next 2 days was still further improved without the use of more powder, partly, at least, in virtue of the more favorable consistency imparted to the diet by the meal and owing to the fact that the powder continued to act for at least 1 day after its administration had been discontinued. When the powder was again added there was a second improvement, the utilization of protein reach-

<sup>16</sup> There has been some controversy between the Council on Pharmacy and Chemistry of the American Medical Association and other competent authorities regarding the digestive merits of this preparation. Its use was first suggested to one of us by results obtained in the laboratory of Professor S. R. Benedict before we knew anything of this controversy and it was tried with Dog VIII with apparently beneficial results. The powder was purchased at several different drug stores in New York City and the experiments here described were done without the knowledge of the manufacturing company.

<sup>17</sup> Since the reaction of the alimentary tract then was undoubtedly alkaline, the enzyme responsible for this improvement must have been trypsin. The contention that trypsin cannot exist in such a powder in association with pepsin seems, therefore, to be conclusively answered by this experiment.



ing 91 per cent and that of fat 90 per cent for a period of 5 days. The dog made a distinct gain in weight during this period.

Our conclusion from this experiment is that, while the dog without a stomach has no difficulty in maintaining nitrogen balance, the utilization of food is considerably impaired by the absence of pepsin-hydrochloric acid digestion. With the aid of artificial digestion this was improved sufficiently to insure a satisfactory state of nutrition before pancreatectomy.

*Influence of the Previous State of Nutrition upon the Effects of Pancreatectomy Following Gastrectomy.*

The pancreas was removed from Dogs XV and XVI on the same day (February 15, 1916). As a further test of the influence of the previous state of nutrition on the effects of pancreatectomy, Dog XVI, a poor feeder at best, had fasted completely 1 week previous to this operation. The two dogs therefore presented a strong contrast in nutritive condition.

The respiratory metabolism was determined on Dog XV on the 2 days just preceding pancreatectomy—the last time, in fact, just a few hours before the operation (Table V). On Dog XVI it had been determined 1 week before and again the day before operation (Table VI). The two determinations on Dog XV agreed well as regards both the heat production and the respiratory quotients, which were those ordinarily obtained following a mixed diet. With Dog XVI there naturally was a decline in both heat production and respiratory quotients following the fast and it is from the latter level that changes in heat production subsequent to the operation are calculated.

Beginning on the day following pancreatectomy the two dogs were observed side by side, one being placed in the respiration incubator (34) in the morning and the other in the afternoon, for a week. Neither received any food for this period. Urines were saved and analyzed for the respiration periods and the blood was examined for its content of glucose.

Dog XV, presumably with a plentiful supply of glycogen, developed at once a rather severe glycosuria, presenting a G:N ratio of 2.8 on the 1st day. This, however, did not persist long and for the week the average was only 1.7. Dog XVI excreted

a small amount of sugar on the 2nd day and then no more until the 5th. The contrast runs along similar lines as regards the blood sugar and heat production. Dog XV shows high blood sugar and a maximum increase in heat production of 36 per cent on the 2nd day, while Dog XVI showed no abnormal blood sugar until the 4th day and an increased heat production which reached a maximum of 24 per cent on the 6th day. It seems clear, assuming as we may, practically identical surgical results<sup>18</sup> in both animals, that the previous nutritive condition is an important factor. The dog that was kept in good condition by artificial digestion developed all the symptoms (save one) of a tolerably severe diabetes, high blood sugar, moderately high G:N ratio, and large increase in heat production, while the dog that had been fasted developed the disease much more slowly and it did not reach so high a degree of severity.

The matter is a not a simple one, however, for Dog VII, which could not have been so well nourished for the 11 days that elapsed between gastrectomy and the completion of pancreatectomy, exhibited a much more severe glycosuria than did Dog VIII, which was carefully fed and lost but little weight. Moreover, Dogs I to VI were all in good nutritive condition. Finally, neither Dog XV nor Dog XVI developed a respiratory quotient which would indicate the loss of the power to burn glucose and they reacted very much alike in this respect. The average R. Q. for Dog XVI after 1 week of fasting is 0.74; the average for twelve determinations after pancreatectomy is 0.74 (nearly), while for Dog XV it is 0.73. These are quotients ordinarily obtained in starvation. If the respiratory quotient is a criterion of the capacity to oxidize glucose these dogs had this power, albeit of limited range, as long as they lived. Dog XVI died on the 11th day, Dog XV on the 17th day after pancreatectomy.

Unusual care was taken in all of these experiments to remove every shred of pancreatic tissue and in no instance was any such tissue found at autopsy. There have been no exceptions to the general rule in this work. Every dog that has been successfully operated has exhibited the typical effects to a greater or less degree.

<sup>18</sup> It is possible that a little more gastric mucosa was left in Dog XV than in Dog XVI. See note 13.

TABLE V.  
Dog XV. Gastrectomy January 3. Pancreatectomy February 15.

Date.	Weight. kg.	Time.	G per hr.	N per hr.	G : N	Blood sugar. per cent	Respiratory metabolism.				Calories per hr.	Increase in meta- bolism. per cent	Remarks.
							CO <sub>2</sub> per hr.	liters	O <sub>2</sub> per hr.	R. Q.			
Feb. 14.....	10.30	11.18-12.18		0.16			3.57	3.95	0.90		19.19		Basal metabolism.
" 15.....	10.35	12.18- 1.18					3.78	4.60	0.82		21.95		Basal metabolism
		10.45-11.45		0.19			3.52	4.42	0.79		20.89		just before pan-
Average...		11.45-12.45					3.26	4.00	0.82		18.90		createctomy.
							3.53	4.24	0.83		20.23		
Feb. 16.....	9.60	1.30	0.89	0.31	2.8							+26	Fasting throughout
		1.45- 2.45					4.02	5.43	0.74		25.16		with exception
" 17.....	9.45	2.45	0.62	0.30	2.0	0.28	4.12	5.62	0.73		25.89		noted below.
		4.05	0.43	0.31	1.4								
		2.33- 3.33					4.25	0.04	0.70		27.60	+36	
		3.33- 4.33					4.24	5.85	0.73		27.61		
		4.48	1.12	0.36	3.2								
" 18.....	8.00	1.30	0.47	0.36	1.6	0.25							
		2.00- 3.00					3.04	5.54	0.71		25.48	+21	
		3.00- 4.00					3.78	5.10	0.74		23.59		
		4.07	0.63	0.30	2.1								

Feb. 19 .	8 80	1 05 2.40- 3.40 3.40- 4 40 4 55	0.23	0 24	1 0	0.26	3.45 3.57	4.61 4.95	0.75 0.72	21.69 22 89	+10	
" 20. .	8 65	10 15	0.26 0.16	0.23 0.19	1 1 0 87							
" 21	8 30	2.05 2 42- 3 42 3 42- 4 42 5 05	0 36	0 21	1.6	0 30	3.34 3 32	4.61 4.57	0.73 0 73	21.23 21.06	+ 4.5	
" 22. .	8 15	10 20	0.50 0 44	0 26 0.31	2 0 1.4							
" 23 .	8 00	10.15 3 00- 4 00 4.00- 5.00	0.47	0 25	1 9	0 27	3 39 3.79	4 62 5 11	0 74 0 74		*	After administration of sugar by stom- ach.
" 24. . .	7.80											
" 25. . .	7.55	10.40	0 16	0 21	0 8							

\* Dog urinated in cage.

Autopsy.—Mar. 2. No pancreatic tissue.

TABLE VI.  
Dog XVI. Gastrectomy January 7. Pancreatectomy February 15.

Date.	Weight. kg.	Time.	G per hr.	N per hr.	G : N	Blood sugar. per cent	Respiratory metabolism.				In- crease. per cent	Remarks.
							CO <sub>2</sub> per hr.	O <sub>2</sub> per hr.	R. Q.	Calories per hr.		
							liters	liters				
Jan. 26.....	10.85	10.30-11.30					2.76	3.64	0.76	17.08		Basal.
		11.30-12.30					2.97	3.97	0.75	18.61		
Feb. 14.....	9.45	2.09-3.09		0.10			2.40	3.33	0.72	15.38		After fasting 1 week.
		3.09-4.09					2.69	3.54	0.76	16.44		
Average fasting metabo- lism .....										15.91		
Feb. 15.....	9.00	10.57-11.51	0.0				2.76	3.80	0.73	17.66	11	Pancreas removed.
" 16.....		11.51-12.51	0.0				2.70	3.73	0.73	17.51		Fasting throughout.
		1.02	0.0	0.12	0.0							
" 17.....	9.00	9.35	0.07	0.14	0.52							
		10.54-11.54					3.13	4.31	0.73	19.98	25	
		11.54-12.54					3.52	4.79	0.74	22.28*		
		1.00	0.0	0.18	0.0							
" 18.....	8.85	10.00	0.0	0.13	0.0							
		10.25-11.25					2.67	3.77	0.71	17.55	11	
		11.25-12.25					3.01	4.10	0.73	19.15*		
		12.30	0.0	0.11	0.0	0.15						

" 19.....	8.55	10.00	0.0	0.11	0.0		3.05	4.03	0.76	18.92	} 16
		10.26-11.26							0.79	17.96	
		11.26-12.26				0.24					
" 20.....	8.40	12.35	0.0	0.11	0.0						
" 21.....	8.10	10.00	0.17	0.14	1.1						
		10.00	0.30	0.17	1.8						
		11.52-12.52					3.15	4.28	0.74	19.92**	} 24
		12.52-1.52					3.09	4.20	0.73	19.52	
		2.00	0.32	0.16	2.0	0.24					
" 22.....	7.80										
" 23.....	7.60	10.05	0.20	0.15	1.3	0.35					
		11.38-1.05					7.21	9.65	0.75†		
		1.05-2.12					4.68	6.58	0.71†		
" 24.....		12.30				0.27					

After giving glucose  
by vein and  
NaOH.

\* Somewhat restless.

\*\* Dog quiet.

† Dog vomited and urinated.

‡ Probably too low.

Feb. 26. Dog died.

Autopsy.—No pancreatic tissue.

## SUMMARY.

1. The hypothesis upon which this work is based is that the hydrochloric acid produced in the stomach and left unneutralized in the duodenum after pancreatectomy is absorbed by the portal system and poisons the liver, causing the rapid onset of the resulting diabetes in well nourished animals.

2. Three dogs in good nutritive condition, that had the pylorus ligated at the time of pancreatectomy, excreted no sugar during the first 24 hours, owing to the absence of acid in the duodenum.

3. Five dogs with a previous gastrectomy, upon removal of the pancreas, developed little or no glycosuria; but one other dog, when given artificial digestion and kept in good nutritive condition so that a considerable amount of glycogen was probably stored following gastrectomy and previous to pancreatectomy, developed a tolerably severe diabetes, exhibiting high blood sugar, an average G:N ratio of 1.7, and an increased heat production up to 36 per cent.

4. The respiratory quotient of this dog and of two others previously gastrectomized was but little lower than that of ordinary starvation.

5. Dogs with pancreas removed after gastrectomy do not exhibit the usual profound toxemia of a simple pancreatectomy.

The results of this study taken in conjunction with the results of Rona (35) and his associates, showing the extreme sensitiveness of the mechanism for oxidation of glucose to the concentration of hydrogen ions, and the work of Murlin and Kramer demonstrating the beneficial effect of alkali administered to totally and partially depancreatized dogs on the oxidation of glucose, suggests that the internal function of the pancreas may be closely akin to its external function in that, on the one hand (external), it provides for the complete neutralization of the acid contents of the stomach, thereby protecting the liver in its glycogenic function, and on the other (internal), it somehow preserves the proper concentration of hydrogen ions in the tissues for combustion of glucose. It is not impossible that the pancreatic hormone, through which this latter function is discharged, may prove to be a peculiarly adapted alkali produced by the islands of Langerhans.

A differentiation of cells for the production of alkali in the pancreas is *a priori* no more improbable than such a differentiation for the production of acid in the stomach. The alkali which passes into the intestine neutralizes the hydrochloric acid of the stomach, but it may also be given off to the blood. Acidity of the tissues may be due to the accumulation of carbon dioxide. A peculiarly adapted alkali which should maintain the combining power of the blood (corpuscles as well as plasma) for carbon dioxide would serve to maintain the normal reaction in the tissues.

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# PRELIMINARY OBSERVATIONS ON THE INFLUENCE OF SODIUM CARBONATE, ADMINISTERED BY DUO- DENAL TUBE, UPON HUMAN DIABETES.

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There are many points of difference between experimental pancreatic diabetes in the dog and human diabetes mellitus. These have been tersely stated by Falta (1) and Allen (2). There has never been any serious question, however, of their fundamental identity. In both, the primary defect is inability to oxidize glucose, as proved by the low respiratory quotient, and the elimination of this sugar *in toto* when ingested.

In view of the demonstrated effects of alkali upon the glycosuria and upon the respiratory metabolism of diabetic dogs (3), the question has presented itself as to whether the failure to observe favorable effects in man has been due to inattention to details or to the administration of alkalies either too strong or too weak. Evidence has been cited in a previous paper (4) that a very slight absolute change in hydrogen ion concentration might make a great difference in the capacity of exsected organs to utilize glucose, but it is well known that the hydrogen ion concentration of the blood, and still more of the tissues, cannot be easily altered. However this may be, the prevailing opinion (5) amongst clinicians has been that alkali affects only the acidosis.

There have been, it is true, some dissenters from this view. Without attempting to give credit to all who have recognized the possibility of a direct benefit to the fundamental defect through control of the acidosis, we shall mention a few who have reported significant results. Sawyer (6) observed that alkaline lavage of the stomach was of distinct benefit.

Dietrich (7) found out of forty cases of diabetes in Petrograd only 25 per cent with normal or apparently normal gastric conditions. He likewise reports complete disappearance of the sugar from the urine in the cases to which he applied alkaline lavage of the stomach, even without enforcing an anti-diabetic diet. Funck (8) is certain that in many diabetics stomach disorders play an important part and believes that cures of diabetes effected by treatment of stomach and intestinal trouble justify the general hypothesis of a causal relation between these disorders and diabetes itself.

Menyhért (9) has for many years treated diabetes on the theory that the underlying cause is a disturbance in the balance between secreted ferments and ferments not secreted and a "pathological chemism of the intestinal tract." The first step in his treatment, which according to all accounts available (10) is very effective, consists in the thorough alkalization of the intestine by giving sodium bicarbonate in 0.5 gm. of keratin-covered capsules up to a maximum of forty to fifty pills a day. The balance between ferments he attempts to restore by the use of papain pills. Menyhért claims that diabetics who died in coma have shown an extremely acid condition of the intestinal contents and of the chyle in the thoracic duct. He claims to have depancreatized a dog completely and kept him alive for 8 months under this treatment.

Weiland (11) has clearly recognized, though he has not demonstrated, the importance of alkali in connection with the oatmeal cure, and Underhill (12) has recently reported the apparently beneficial effect of large doses of alkali on a severe glycosuria.

The report of Menyhért's work first suggested to us the importance of administering alkali to diabetics directly into the intestine. Encouraged by the results already reported on dogs, especially from intravenous infusion of alkali, it was resolved to make a critical study on a few selected cases of the effects of alkali administered in different ways. Through the courtesy of Professor Lusk and Dr. Du Bois in placing at our disposal the metabolism ward of the Russell Sage Institute of Pathology with its equipment in Bellevue Hospital, we have been enabled during the past summer to make a study of a half dozen cases.<sup>1</sup>

It was necessary to have perfect control of the diets.<sup>2</sup> All urines and stools were collected quantitatively and properly preserved. Because we wished to follow the effects upon the blood

<sup>1</sup> A portion of the expenses of this research has been defrayed out of the Johnston Livingston Fund for Experimental Medicine.

<sup>2</sup> Miss M. Duggan, who has had 2 years' experience under Dr. Du Bois, was placed in charge of this work, and with the assistance of an additional day and night nurse she has weighed and calculated the components in every particle of food eaten by these patients.

sugar and to note its relation to the  $\text{CO}_2$ -combining power, blood samples were taken, as a rule, every other day.

The 24 hour urine periods closed at 5 a.m., breakfast came at 6, and the blood samples were taken at 11, or between 11 and 11.30. Alkali ( $\text{NaHCO}_3$ ) was administered to one patient by mouth, and to one patient (carbonate as well as bicarbonate) also by intravenous infusion; but the introduction of these solutions directly into the duodenum by means of Einhorn's duodenal tube (13) proved much more satisfactory. We expected two distinct advantages from this mode of administration: (1) there is little danger of vomiting and no interference with digestion; and (2) the alkali would not stimulate the stomach to produce more acid (14). It was to make sure that digestion was not seriously interfered with that the feces were saved and separated into periods of 3 to 6 days, corresponding to the periods of alkali treatment and the intervals between them. Separation of feces was accomplished by means of carmine given in 5 grain capsules. With one patient charcoal was given in the same way.

Once the patient was safely established on a diet of known composition and a control period of several days had been secured for both the glycosuria and the digestion (feces), alkali was given once each day for several days in succession. This was followed by an interval of like extent and then the alkali was resumed. With only three patients were we able to continue these periods on and off alkali as long as we wished. The other three were treated only once or twice.

In the maintenance of a constant diet it was of course recognized that spontaneous fluctuations of the sugar output would occur, and it was expected that in some cases the simple restriction of the carbohydrate would lead to marked improvement. What we hoped to see, however, was a fluctuation down and up coinciding with the periods of alkali and no alkali. Such a tendency is distinctly visible in practically all the cases.

Two diets, one containing approximately 15 gm. of carbohydrate and the other approximately 100 gm. have been chiefly used. The articles of diet were selected with a view to uniformity of composition. Thus the meat was all lean beef ground, or chicken carefully selected and separated from skin, gristle,

etc. The milk, butter, bread, peas, and oatmeal used had been analyzed by Dr. Gephart in connection with the metabolism work in the hospital conducted by Professor Lusk and Dr. Du Bois. We found it necessary, therefore, to make but few analyses for this research.

### *Methods.*

The method of administration of the alkali deserves special mention. The same method had been used independently by Dr. Du Bois in the administration of alkali to nephritics. The Einhorn tube was given to the patient about 8 or 9 a.m. It is easily swallowed with the help of a little water. By having the patient lie upon his right side the "bucket" would in most cases be found in the duodenum in a couple of hours. As soon as possible after drawing the blood at 11 a.m., the alkali, heated to about 45°C., was allowed to run into the intestine from a thermos bottle used as a Mariotti flask, by the visible drip method. In the majority of instances we have used 500 cc. of fluid and have adjusted the flow at such a rate that it required an hour to enter. Without exception the patient has been able to eat dinner as soon as all the alkali was in the duodenum. In a few instances larger amounts of fluid were used and proved too much for the bowel to hold. Several loose stools within a couple of hours resulted.

The methods of analysis applied to the urine and blood were those which have been employed in this laboratory in the studies of pancreatic diabetes in the dog (3). Only the method of obtaining the CO<sub>2</sub>-combining power of the blood deserves special mention. The chemical method of Haldane (15), employing the monometers devised by Brodie (16), has been used. We have chosen the entire blood rather than the plasma, partly because it is much more convenient to transfer whole blood to such an apparatus, and partly because there is some reason to believe that the combining power of hemoglobin as well as of the plasma for CO<sub>2</sub> is dependent upon the amount of alkali present (17). Finally we wished especially to investigate possible relations between the CO<sub>2</sub>-combining power and the blood sugar, and since the blood sugar occurs in the corpuscles as well as in plasma the whole blood should be used in both determinations. We shall go into this subject more fully in a later paper.

5 to 7 cc. of blood were drawn into a Brodie pipette and mixed with 0.3 cc. of hirudin solution. 0.5 cc. was then transferred to the bottle of the Haldane-Brodie monometer and while in this bottle was saturated with  $\text{CO}_2$  by blowing alveolar air directly onto the blood, the bottle meanwhile being rotated rapidly. Saturation is accomplished in this fashion in 2 to 3 minutes. Withdrawing the glass tube through which the breath is directed into the bottle and quickly closing the neck of the bottle with the thumb, the operator can attach the bottle, with blood and alveolar air contained, to the monometer without loss. After equalization of temperature in the several bottles (duplicates for each sample) and the control monometer, the regular Haldane method is carried out, driving off oxygen first.

Control analyses were made in the same manner in every detail on several normal bloods, and often both kinds of blood were analyzed on the same day, saturation being accomplished with the same alveolar air in the several samples within a few minutes of each other. In all the charts and tables which follow, the figures for the blood were obtained from samples taken at the beginning of the urine day. In other words, the blood sugar level and the  $\text{CO}_2$ -combining power which prevailed at or near the beginning of the 24 hour period and which must have influenced the output of sugar for this period are shown.

### *Description of Cases and Results.*

The first case that we encountered proved to be an interesting one of renal glycosuria. Since the case was not suitable for treatment with alkali, it will be reported elsewhere. The fourth case also was one of peculiar interest because it was soon learned that the patient had little or no pancreatic digestion. This case will serve, however, to introduce the subject of the influence of alkali and will be considered here (Case 1).

A brief description of the cases will suffice, because the tables show the degree of severity presented.

*Case 1.*—H. H., an extremely emaciated man, age 55 years; no teeth; arteries slightly thickened; physical examination otherwise negative. Family history negative for tuberculosis and diabetes. Had had scarlet fever and measles as a child, gonorrhea 25 years ago. Otherwise well and

TABLE I.

Case 1. H. H.

Date, day ending.	Weight. kg.	Food.				Urine.					Blood.		Remarks.
		Total calories.	Car- bohy- drate.	Fat.	Food N.	Vol- ume.	Sp. gr.	Acidity 0.1 N.	NH <sub>3</sub> N	Total N.	Glucose.	Sugar	
		gm.	gm.	gm.	gm.	cc.		cc.			per cent	Sp. gr.	
July 3, 11 a.m.		1,633	50.5	123.5	11.2	3,035	1.022	569	1.09	11.47			
" 4.....	42.6	1,633	50.5	123.5	12.2	2,357	1.025	613	1.27	10.56			
" 5.....	42.5	1,571	14.9	129.4	12.9	1,785	1.014	683	1.58	12.30	2.62	0.09	
" 6.....	42.3	1,610	14.9	132.8	12.4	4,076	1.011	1,039	2.20	19.06	0.0		
" 7.....	41.2	1,652	14.9	136.8	13.3	2,780	1.011	737	1.63	14.6	0.0	0.08	1.069
" 8.....	40.8	1,608	14.9	132.3	13.3	3,970	1.009	794	1.86	17.17	0.0		5 gm. digestive
" 9.....	37.9	1,608	14.9	132.3	13.6	2,355	1.011	489		14.97	0.0		powder after
" 10.....	37.1	1,608	14.9	132.2	13.6	1,845	1.012	452		12.97	0.0		each meal.
" 11.....	37.2	1,485	30.3	104.1	16.2	1,525	1.012	412		13.28	0.0	0.08	
" 12.....	37.9	1,652	30.3	126.1	14.2	1,925					0.0		
" 13.....	37.8	1,652	30.3	126.1	14.8	2,905	1.007				0.0		
" 14.....	37.9	1,511	50.2	127.1	12.9	3,430	1.008			16.81	0.0		
" 15.....	35.7	1,709	50.2	127.2	13.4	2,710	1.010			14.98	0.0		
" 16.....	36.0	1,626	50.2	122.9	12.1	2,562	1.012			15.49	0.0		
" 17.....	35.3	1,646	50.2	122.9	11.3	3,020	1.008			12.01	0.0		
" 18.....	34.6	1,641	50.2	123.3	11.6	1,590	1.010			13.32	0.0		10 gm. digestive
" 19.....	35.1	1,636	49.2	123.4	11.6	1,983	1.011			13.52	0.0	0.141	powder (lacto-
" 20.....	35.6	1,631	50.2	123.4	11.6	1,670	1.013	251		11.50	4.54		peptine) before
" 21.....	35.8	1,631	50.2	123.4	11.6	1,375	1.013			11.51	8.91	0.270	dinner in 0.3
" 22.....	35.6	1,641	50.2	123.4	11.2	1,690	1.014	211		10.08	11.51		per cent Na <sub>2</sub> - CO <sub>3</sub> by duo-

July 23.....	35.5	1,628	50.2	123.0	11.2	1,190	1,014	184				9.3	6.28	5 gm. lactopep- tine before each meal. No al- kali.
" 24.....	36.2	1,628	50.2	123.0	12.1	1,925	1,013	231				11.97	11.90	
" 25.....	36.1	1,641	50.2	123.4	11.6	1,465	1,016	227				9.48	15.65	
" 26.....	37.1	1,641	50.2	123.4	12.1	1,598	1,014	244	1.28	8.95	12.11	8.95	12.11	10 gm. lactopep- tine in 300 cc. 0.3 per cent Na <sub>2</sub> CO <sub>3</sub> once daily.
" 27.....	37.5	1,638	50.2	123.0	11.7	1,945	1,009	248	1.27	8.28	4.46	8.28	4.46	
" 28.....	37.8	1,716	50.2	129.7	12.8	1,920	1,009	250	1.09	7.31	0.0	7.31	0.0	
" 29.....	37.8	1,716	50.2	129.7	12.7	2,202	1,010	264	1.03	7.21	0.0	7.21	0.0	No alkali. No digestive pow- der.
" 30.....	37.2	1,716	50.2	129.7	12.6	2,100	1,011	399	1.12	8.58	0.0	8.58	0.0	
" 31.....	37.2	1,716	50.2	129.7	12.6	1,990	1,011	458	1.18	11.03	0.0	11.03	0.0	
Aug. 1.....	38.3	2,004	100.2	132.7	14.4	1,965	1,011	462	1.05	10.95	7.22	10.95	7.22	5 gm. lactopep- tine 20 min. be- fore each meal.
" 2.....	36.1	1,950	93.6	131.9	13.6	2,460	1,013	320	1.38	9.44	21.02	9.44	21.02	
" 3.....	36.0	1,950	93.6	131.9	13.2	1,935	1,016	242	1.23	8.56	31.48	8.56	31.48	
" 4.....	36.1	1,950	93.6	131.9	13.6	1,983	1,017	288	1.28	8.94	33.74	8.94	33.74	
" 5.....	36.3	1,980	100.3	131.0	13.6	2,530	1,017	291		9.78	42.8	9.78	42.8	10 gm. lactopep- tine before each meal. No al- kali.
" 6.....	36.3	1,980	100.3	131.0	13.6	1,977	1,018	237		10.18	46.5	10.18	46.5	
" 7.....	36.6	2,013	100.3	133.4	14.0	1,905	1,019	257		8.96	44.0	8.96	44.0	
" 8.....	38.0	2,003	100.3	132.4	14.5	2,530	1,020	304		9.28	65.3	9.28	65.3	
" 9.....	38.1	2,003	100.3	132.4	14.5	2,910	1,016	276		9.94	57.5	9.94	57.5	
" 10.....	38.2	2,003	100.3	132.4	14.5	2,520	1,017	233		8.54	47.0	8.54	47.0	10 gm. lactopep- tine before each meal, once in alkali 0.3 to 0.5 per cent by duodenal tube.
" 11.....	38.2	1,990	100.3	132.0	14.1	2,335	1,020	257		8.24	49.4	8.24	49.4	
" 12.....	38.6	1,990	100.3	132.0	14.1	2,580	1,020	271		8.38	51.8	8.38	51.8	
" 13.....	38.4	2,003	100.3	132.4	14.5	2,682	1,017	302		8.64	46.5	8.64	46.5	
" 14.....	39.0	2,003	100.3	132.4	14.1	2,665	1,019	280		9.10	53.1	9.10	53.1	
" 15.....	38.8	2,003	100.3	132.4	14.5	2,925	1,018	322		9.50	55.0	9.50	55.0	



TABLE I—Concluded.

Date, day ending.	Weight. kg.	Food.					Urine.					Blood.		Remarks.
		Total calories.	Car- bohy- drate.	Fat.	Food N.	Vol- ume.	Sp. gr.	Acidity 0.1 N.	NH <sub>3</sub> N	Total N.	Glu- cose.	Sugar.	Sp. gr.	
		gm.	gm.	gm.	gm.							per cent		
Aug. 16.....	39.1	2,004	100.3	132.4	14.5	cc.	1.017	302		8.90	28.7			No digestive pow- der and no al- kali.
" 17.....	38.8	2,003	100.3	132.4	14.7	2,515	1.015	278		5.27	14.4			
" 18.....	39.2	1,990	100.3	132.0	14.1	2,390	1.011	354		6.16	12.6			
" 19.....	39.7	2,003	100.3	132.4	14.4	2,210	1.010	363		6.66	5.4			
" 20.....	39.6	2,003	100.3	132.4	14.4	2,344	1.010					0.139	1.054	5 gm. Fairchild's extract of pan- creas before meals, 1 day only.
" 21.....	38.9	2,003	100.3	132.4	14.4	3,060	1.010	337		8.14	0.0			
" 22.....	39.1	1,952	100.3	128.6	13.8	1,920	1.011	355		7.31	2.4			
" 23.....	38.9	1,952	100.3	128.6	13.8	3,055	1.008	321		7.87	0.0			
" 24.....	38.4	1,952	100.3	128.6	13.8	2,855	1.009	357		7.55	0.0	0.083	1.052	5 gm. Merck's pancreatin 20 min. before each meal every day.
" 25.....	37.4	1,952	100.3	128.6	13.5									
" 26.....	37.0	1,952	100.3	128.6	13.1	2,830	1.018			9.67	38.7			
" 27.....	37.6	1,952	100.3	129.0	14.2	2,610	1.013			8.48	23.5			
" 28.....	38.2	1,952	100.3	128.6	13.5	2,555	1.014			10.44	28.2			
" 29.....	38.0	1,966	100.3	129.0	13.8	2,885	1.018	403		11.39	48.0			
" 30.....	37.7	2,266	123.8	144.6	16.1	3,105	1.018	342		12.26	62.9			
						1,975	1.024	384		12.02	52.4			

strong up to present illness. Had been a rather heavy drinker—fifteen to twenty whiskies daily for 10 to 12 years. None now for 2 years, but takes beer occasionally. Present illness began  $2\frac{1}{2}$  years ago (1914). He felt tired and run down, and examination of urine revealed sugar. Took medicine and dieted, but gradually "went down hill," losing weight. Gave up dieting last fall (1915). Stopped work on June 1 on account of weakness. Has lost 60 pounds in  $2\frac{1}{2}$  years. Eyesight and hearing are rapidly failing. Legs becoming numb, marked dyspnea on exertion. Occasional swelling of hands and feet.

This patient when placed on low carbohydrate immediately became sugar-free, but instead of improving lost weight rapidly while eating more than 40 calories per kg. daily. In 2 weeks he had fallen off fully 8 kg. Examination of the stools during this time revealed the fact that he had practically no pancreatic digestion. The stools were very light in color and like butter, being very hard while in the ice box and soft and semifluid when at room temperature, in which state the fat could be seen floating on the surface. They also contained large amounts of undigested meat, egg, etc.

Careful palpation of the abdomen at this time disclosed a very hard and resistant body just above the umbilicus extending transversely from  $1\frac{1}{2}$  to 2 inches to the right of the median line, which was thought to be the pancreas much hardened and atrophied.

Artificial digestion was instituted with lactopeptine powder on July 7. For the first 3 days it was given  $\frac{1}{2}$  hour after each meal, but it did not appear to be beneficial. The bowels continued loose and the stools copious. Artificial digestion was discontinued until the 16th, when the powder was given in 0.3 per cent sodium carbonate by means of the duodenal tube. Following the 3rd day of this treatment sugar began to appear in the urine, indicating that digestion of starch was taking place. Since the infusion was given just before dinner each day, this suggested that the pancreatic enzymes would better survive the stomach if given before meals. Accordingly on the 22nd 5 gm. were given in water 20 minutes before each meal, and the fact that the trypsin in the powder actually survived the stomach was proved by recovery of it from the duodenum.<sup>3</sup>

<sup>3</sup> Since this is a rather important practical point for the administration of pancreatic digestion artificially, some details of these tests will be given. On August 1, just before giving the powder on an empty stomach, the duo-

There are two points of particular interest in this case. (1) The urine contained no sugar as long as the digestion was not assisted artificially, but soon after the digestive powder was given, sugar appeared. The amount of sugar varied (a) with the amount of carbohydrate in the food (see table) and (b) with the amount of digestive powder administered (see Fig. 1.) With 100 gm. of carbohydrate in the food, as much as 65 gm. of glucose were found in the urine. That the other 30 gm. were being used by the patient is not certain, although it is probable from the fact that he gained weight during the period. (2) While alkali (0.3 per cent  $\text{Na}_2\text{CO}_3$ ) was being given with the digestive powder the sugar disappeared from the urine (July 28). The strength of alkali was such as not to interfere with pancreatic digestion; hence it is probable that the carbohydrate was better utilized under the influence of the alkali. Again, when the amount of digestive powder was increased and digestion had reached its maximum, the addition of alkali brought the excre-

denal tube being in place (as proved by the viscid yellow contents with strong alkaline reaction), one-third of a syringe-full of water was introduced and one-half syringe-full was immediately withdrawn. This fluid, strongly tinted with bile, was set aside in Test-tube 1. A half glass of water, containing 5 gm. of lactopeptine (New York Pharmacal Association), was then taken by the patient and 10 minutes later a second sample was taken from the duodenum in the same manner (Test-tube 2). A third was drawn at the end of 20 minutes. In all these samples the powder was clearly visible. During the time since drinking the water the patient was required to lie upon his right side in order to facilitate emptying the stomach.

The three samples of duodenal contents were tested by placing 2 cc. of each fluid in 5 cc. of 0.3 per cent  $\text{Na}_2\text{CO}_3$ , and adding about 1 gm. of stained fibrin. Incubation at  $38^\circ\text{C}$ . Pancreatic digestion was very evident in Test-tube 3 (containing duodenal contents drawn 20 minutes after ingestion by stomach) at the end of 10 minutes' incubation. At the end of 30 minutes digestion was evident also in No. 2, but continued much stronger in No. 3. At the end of  $3\frac{1}{2}$  hours the fibrin in No. 3 was all disintegrated, that in No. 2 was much broken up, but no digestion at all had taken place in No. 1.

Similar tests were carried out on two other patients (N. P. and M. P.) who had normal gastric and pancreatic digestion. The only difference in result was that the first sample (drawn before ingestion of the powder) showed some digestion, but not so much as the other samples containing the powder freshly passed through the stomach.

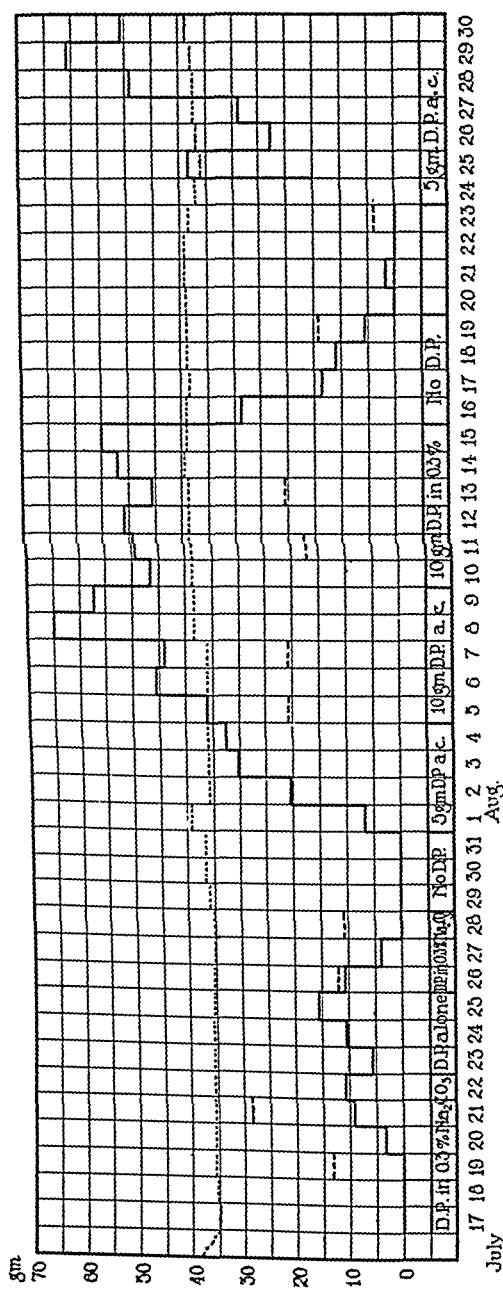


FIG. 1. Case 1. — Gm. of sugar in urine. --- Hundredths of a per cent blood sugar. .... Body weight in kg. D. P. denotes digestive powder; a. c., before meals. Lactopeptine was used up to August 24, Merck's pancreatin thereafter.

tion of sugar down sharply and at once, and held it at a nearly constant level. In the absence of any reason to believe that digestion was interfered with (see examination of feces later) this may be taken as evidence of improved utilization, particularly in view of the continued gain in weight at this time.

When the digestive powder was discontinued the sugar in the urine again fell off promptly but did not wholly disappear for 2 days. During this time the powder must have continued to digest starch in the intestine. Merck's pancreatin was used instead of lactopeptine during the last period. The amount of sugar excreted shows that 5 gm. of this powder is about equal in diastatic power to 10 gm. of the lactopeptine. The latter preparation contains pepsin, but we are not aware of the exact proportions. The biological test here applied indicates that pancreatin may constitute from 40 to 50 per cent of its weight.

The blood sugar in this case varies always with the amount of pancreatic digestion. The specific gravity shows that such fluctuations are not due to variations in dilution when alkali was administered.

*Case 2.*—J. R., a poorly nourished, slightly anemic man, age about 30 years; married. Family history negative. No childhood diseases, never sick in bed until present illness, which began suddenly in April, 1915. Worked 14 years as a butcher. Drank moderately. Had had as high as 7 or 8 per cent of sugar in urine. Present weight 115 pounds, normal weight 178. Had been to Mt. Sinai Hospital in March, 1916, at which time fasting for 1 or 2 days had rendered the urine sugar-free. Referred by Dr. A. I. Ringer.

Placed on 15 gm. of carbohydrate, this patient excreted after the 1st day nearly 100 gm. of carbohydrate, and his G:N ratio was 4.0. On this same day he showed symptoms of severe acidosis (high acidity in urine, acetone breath, headache, red tongue) and bicarbonate of soda was started in 10 gm. doses. For 3 days 30 gm. were given daily and there was by the end of this time a marked general improvement. While the total sugar in the urine had not gone down consistently the sugar in the blood had yielded and the G:N had dropped. Then for 3 days 900 cc. of Ringer's solution to which had been added sodium bicarbonate in the amounts noted in the table, were infused through the Einhorn tube into the intestine. The sugar in the urine gave signs

TABLE II.  
Case 2. J. R.

Date.	Weight. kg.	Food.					Urine.					Blood sugar by weight.	Sodium bicarbonate.
		Total calories.	Car- bohy- drate.	Fat.	N.	Vol- ume.	Sp. gr.	Acidity 0.1 N.	Total N.	Glu- cose.	G : N.		
July 5-6.....	48.9	1,764	50.5	131.1	13.9	2,710	1.037	3,496	22.61	134.7		0.26	
" 6-7.....	48.8	1,773	14.9	142.5	15.4	2,605	1.034	1,485	22.54	109.9	4.2	0.262	
" 7-8.....	47.8	1,755	14.9	141.8	15.0	2,850	1.030	1,297	20.91	99.3	4.0		10 per os.
" 8-9.....	48.0	1,755	14.9	141.8	15.0	2,730	1.030	1,010	19.65	93.1	4.0	0.266	30 "
" 9-10.....	48.7	1,755	14.9	141.8	15.0	2,705	1.030	906	20.11	86.3	3.5	0.166	30 "
" 10-11.....	48.6	1,748	14.9	141.2	15.4	3,195	1.030	927	25.68	106.8	3.5		27 by duodenal tube.
" 11-12.....	49.4	1,755	14.9	141.8	14.6	2,935	1.028	822	22.49	85.8	3.1	0.199	54 "
" 12-13.....	50.0	1,773	14.9	142.5	15.4	2,650	1.025	543	18.49	72.3	3.1		27 "
" 13-14.....	51.8	1,773	14.9	142.5	15.4	2,570	1.027	681	17.95	70.2	3.1	0.188	0
" 14-15.....	52.8	1,775	14.9	142.5	15.6	2,330	1.027	699	18.13	64.4	2.7	0	0
" 15-16.....	51.9	1,775	14.9	142.5	15.6	2,540	1.026	787	19.23	48.1	1.7	0.172	0
" 16-17.....	51.4	1,775	14.9	142.5	15.6	2,063	1.027	645	16.75	50.8	2.1	0	0
" 17-18.....	51.2	1,775	14.9	142.5	15.6	2,815	1.024	781	21.52	78.7	2.96	0	0
" 18-19.....	51.4	1,774	14.6	142.5	15.1	2,810	1.023	731	19.20	73.2	3.0	0.184	0
" 19-20.....	50.7	1,791	14.6	143.3	16.2	2,575	1.025	670	20.77	67.5	2.5	0	0
" 20-21.....	50.6	1,791	15.1	143.2	15.9	2,535	1.026	583	17.60	68.7	3.1	0.158	27 "

of falling off at once, but this did not become marked until the 2nd day after the infusion was stopped. A delay of this sort in the effect of bicarbonate as opposed to carbonate has been noted by Murlin and Kramer in the dog (3). The output of glucose then for 2 days was only about one-half what it had been when the alkali was started. Then it began to rise and fluctuated for several days about 70 gm. The G:N ratio at its lowest point was 1.7, from which it rose again to the neighborhood of 3.0. The nitrogen in the urine had also fallen off considerably, giving the appearance of interference with digestion. On account of severe constipation at the start and some diarrhea as the result of these duodenal infusions it was impossible to make accurate separation of the feces in this case.

The blood sugar, after a sudden preliminary drop when the alkali was started, follows (or, preferably, precedes) in a general way the level of the urinary sugar. The specific gravity of the blood was not taken in this case, but to judge by the cases which follow, the fluctuations of glycemia are not due to the dilution of the circulating medium.

*Case 3.*—N. P., a well formed, medium sized man, age about 35 years; married. Physical examination negative. Family history negative for tuberculosis and diabetes. Had no childhood diseases and had never been ill before present illness which began 10 months ago (September, 1915). Had worked as a baker and had eaten much sweet cake by way of testing it. Drank some beer but no whiskey. Onset of diabetes sudden; patient noticed that he was urinating frequently and was losing weight. Family physician diagnosed diabetes and placed him on a diet with carbohydrate not entirely excluded. Lost 50 pounds the first 3 or 4 months, but by careful dieting has held his weight at 143 pounds for the past 6 or 7 months. No increased frequency of micturition at present. Complaints only of weakness.

This patient was kept throughout on 15 gm. of carbohydrate, from 17 to 18 gm. of nitrogen, and about 35 calories per kg. daily. For 5 days on this diet, during which the feces were being saved for control, the glycosuria fell off distinctly, and the first period of alkali (bicarbonate of soda in Tyrode's solution) seemed to aggravate it for the first 2 days. At the end of the period it was at the same level as before the alkali was given (compare Case 2). But within the next 4 days without alkali, there was a

rise in output of about 30 gm. and when 1 per cent sodium carbonate was given another fall of nearly 30 gm. (on the 2nd day) took place. The level then maintained was lower than at any time before.

The second period without alkali was marked by an even greater rise in the sugar, and although this was checked by the first in-

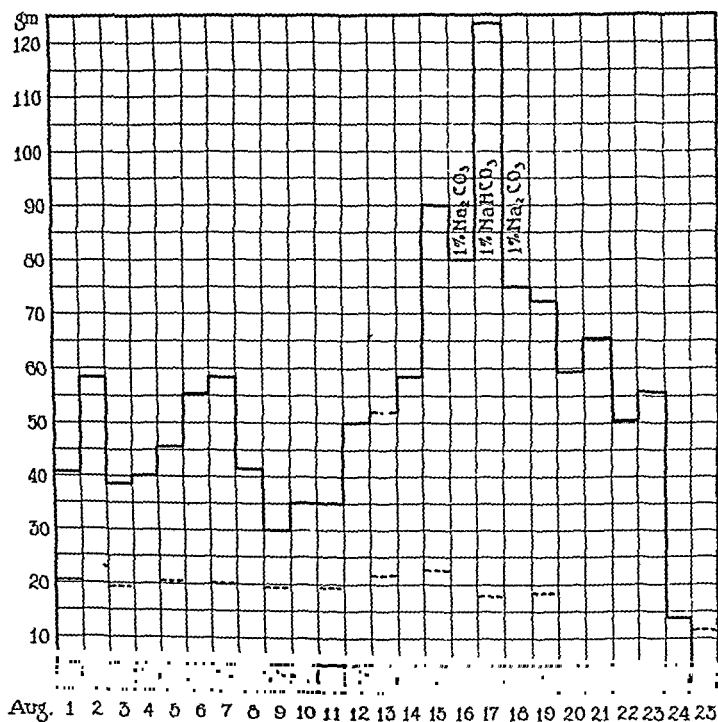


FIG. 2. Case 3. — Gm. of sugar in urine. --- Hundredths of a per cent blood sugar.

travenous infusion of carbonate, on the 2nd day when bicarbonate was used the sugar excretion became very high. Murlin and Kramer (3) have noted this effect of bicarbonate to cause a sudden flushing out of glucose even when the subject, a depancreatized dog, was entirely free of sugar. The contrast with carbonate is very sharp in this case and judging by the volume of urine we must suppose that it is largely a washing out process.



TABLE III.

Case 3. N. P.

Date, day ending.	Weight. kg.	Food.				Urine.				Blood.			Remarks.	
		Total calories.	Car- bohy- drate.	Fat.	N.	Vol- ume.	Sp. gr.	Acidity 0.1 N.	Total N.	Glu- cose.	Sugar.	CO <sub>2</sub> - combin- ing power.		Sp. gr.
July 26.....	58.7	2,358	15.1	186.7	22.8	1,405	1.041	576	17.2	68.8	0.226		1.074	
" 27.....	58.6	2,094	15.1	171.0	18.1	1,710	1.034	838	24.5	49.5				
" 28.....	58.2	2,094	15.1	171.0	18.1	1,402	1.034	792	21.8	43.0	0.172		1.071	
" 29.....	57.5	2,094	15.1	171.1	18.1	1,290	1.035	697	19.2	43.1				
" 30.....			15.1			1,435	1.031	710	19.4	39.7	0.169	71.5	1.064	
" 31.....	57.8	2,095	15.1	171.1	18.1	1,218	1.034	658	16.8	36.2				
Aug. 1....	57.7	2,095	15.1	171.1	17.6	1,580	1.029	498	18.3	42.0	0.208	65.2	1.070	900 cc. Tyrode's so- lution + 3 per cent NaHCO <sub>3</sub> by duodenal tube once daily.
" 2.....	58.0	2,095	15.1	171.1	18.1	1,388	1.037	347	17.9	58.4				
" 3.....	59.0	2,095	15.1	171.1	17.2	1,675	1.029	209	17.9	38.1	0.199	80.6	1.068	
" 4.....	59.2	2,095	15.1	171.1	17.6	1,533	1.037	391	16.1	40.3				No alkali.
" 5.....	58.5	2,095	15.1	171.1	17.2	1,405	1.035	562	17.2	46.5	0.217	72.0	1.081	
" 6.....	58.1	2,095	15.1	171.1	17.2	1,670	1.032	576	17.3	56.7				500 cc.
" 7.....	57.3	2,095	15.1	171.1	17.2	1,630	1.034	587	19.3	58.6	0.201	61.2		
" 8.....	56.9	2,095	15.1	171.1	17.6	1,280	1.036	563	18.2	42.7				Ringer's solution + 1 per cent Na <sub>2</sub> CO <sub>3</sub> by duodenal tube once daily.
" 9.....	57.0	2,095	15.1	171.1	17.6	1,329		520	17.9	30.4	0.199		1.070	
" 10.....	57.3	2,095	15.1	171.1	17.6	1,840		475	17.3	35.9				
" 11.....	57.0	2,095	15.1	171.1	17.6	1,735	1.026	451	16.7	34.9	0.188		1.067	

Aug. 12.....	57.4	2,095	15.1	171.1	17.6	1,835	1.027	560	17.1	50.1	0.225	66.3	1.065	No alkali.
" 13.....	56.8	2,095	15.1	171.1	17.6	1,445	1.035	621	17.7	58.5				
" 14.....	55.6	2,095	15.1	171.1	17.6	2,155	1.035	862	22.8	90.4	0.234	69.7	1.067	
" 15.....	55.9	2,095	15.1	171.1	17.6	2,233		878	25.0	80.1				500 cc. Ringer's solution + 1 per cent Na <sub>2</sub> CO <sub>3</sub> intravenously.
" 16.....	55.3	2,095	15.1	171.1	17.6	2,762		810	26.2	123.8	0.179	71.6		860 cc. Tyrode's solution + 1 per cent NaHCO <sub>3</sub> intravenously.
" 17.....	55.8	2,095	15.1	171.1	17.6	1,836	1.034	707	16.3	75.4				500 cc. Ringer's solution without CaCl <sub>2</sub> + 1 per cent Na <sub>2</sub> CO <sub>3</sub> .
" 18.....	56.2	2,095	15.1	171.1	17.6									
" 19.....	56.1	2,095	15.1	171.1	17.2	1,605	1.036		19.14	73.2	0.183	64.7	1.068	500 cc. Ringer's solution + 1 per cent Na <sub>2</sub> CO <sub>3</sub> by duodenal tube.
" 20.....	55.6	2,095	15.1	171.1	17.2	1,445	1.035	448	17.78	59.3				
" 21.....	55.7	2,095	15.1	171.1	17.6	1,640	1.034	607	20.82	66.6		76.0	1.067	
" 22.....	56.2	2,095	15.1	171.1	17.2	1,572	1.029	432	14.26	52.0				
" 23.....	56.8	1,128	9.0	70.8	18.5	1,535	1.035	614	20.8	56.8				No alkali.
" 24.....	55.3					920	1.039	350	13.79	14.2		76.6	1.067	Fasting.
" 25.....	54.1					1,050	1.016	394		0.0	0.128			
" 26.....	53.7					575	1.032		6.57	0.0				

Comparison of this period with the following one when 1 per cent carbonate was given by the duodenal tube shows that the latter method produces the greater effect. Does this signify that some factor derived from the alimentary wall is at work, or only that the beneficial effect is prolonged by slow absorption? If the acid chyme passing into the duodenum can intensify the diabetes of a depancreatized dog by starting some toxic action at the duodenal wall (4), can we not say that the normal corrective is an antagonistic action of alkali starting at the same point?<sup>4</sup>

The nitrogen of the urine varies with the sugar. The analyses of feces which in this patient were easily separated show that this fluctuation cannot be located in the processes of digestion.<sup>5</sup>

The figures for blood sugar in this case do not run perfectly parallel to the figures for urinary sugar, and yet, with but few exceptions, the tendency to do so is evident. Perhaps if blood samples had been taken every day this would be displayed still better. *The specific gravities show that fluctuations in blood sugar cannot be explained by dilutions.*

The CO<sub>2</sub>-combining power of the blood presents some points of interest. The technique throughout was exactly the same.<sup>6</sup> Normal combining power by the method here employed runs around 85 volumes per cent. The first determinations in this case were made previous to the administration of any alkali. The figures are low enough to denote a distinct acidosis. In the first sample taken after alkali (on the morning following the second infusion) the combining power was nearly normal. Later when alkali was given by vein the combining power was not affected so much. This probably means only that the alkali placed directly in the circulation was more quickly eliminated. When (in the last period) 1 per cent carbonate had been given

<sup>4</sup> The intravenous method is obviously attended with danger of emboli. The median vein was used in this patient, and on the day following each infusion the vein at the site of injection was found filled with an organized clot. Happily these did not cause any serious trouble. The strength of solution was never more than 1 per cent. Judging by this experience the infusion of stronger solutions of alkali must be a really hazardous procedure.

<sup>5</sup> See page 311.

<sup>6</sup> See page 292.

for 3 days by duodenal tube the combining power had risen to 76 and it was again at this level after starvation for 1 day.

*Case 4.*—M. P., a somewhat obese man, age over 50 years; married. Physical examination negative except for skin and toes. On left foot middle toe is reddened and quite edematous at end, ulcerated in front; fourth toe also reddened and swollen. On right foot great toe somewhat edematous; median surface reddened and tender. (The lesions were later pronounced gangrenous.) Skin on palmar surface of hands dry and scaling. Family history negative for tuberculosis and diabetes. Mother died of "Bright's disease." Chicken-pox only childhood disease. General health had been good except for an attack of articular arthritis in 1910. Patient is an elevator man; had been a moderate drinker, taking whiskey before breakfast occasionally.

Present illness came on suddenly about January, 1915, when patient noticed weakness, loss in weight, great thirst, and appetite. Physicians told him he had 4 to 7 per cent sugar. Went on restricted diet for 3 to 4 months and felt much better. In the past year has had no diabetic treatment. Toe on left foot became sore and ulcerated in April, 1916, and in June, toe on right foot. Patient has felt weak ever since. Has lost weight but does not know how much. Urination five to six times daily and two or three times at night.

With the exception of the 1st day, which probably reflects the influence of the previous diet, the sugar excreted during the first period was approximately nine-tenths of the carbohydrate ingested. On the first 3 days on treatment with alkali (bicarbonate through duodenal tube) the glycosuria fell off sharply, but remained stationary or nearly so when the alkali was discontinued. When next it was resumed, different strengths of carbonate were introduced into the intestine with a view to finding the minimal strength which would produce an effect on the glycosuria. The chart shows that this was not reached until 1 per cent was used, whereupon the excretion fell off immediately about 20 gm. Once more the sugar rose when the alkali was interrupted for 2 days, and was brought down to 35 gm. by intermittent doses a day apart.

There is in this case no close correspondence between the level of blood sugar and the glycosuria. The lowest points in the two curves do not correspond and, after the 1st day, the highest points do not. Nevertheless there is marked improvement in the glycemia as well as in the glycosuria. The figures for the CO<sub>2</sub>-combining power show that the alkali persists in the blood for at least 1 day after the infusion.

TABLE IV.  
Case 4. M. P.

Date, day ending.	Weight.  kg.	Food.				Urine.				Blood.			Remarks.	
		Total calories.	Car- bohy- drate.	Fat.	N.	Vol- ume. cc.	Sp. gr.	Acidity 0.1 N.	N.	Glu- cose.	Sugar.  per cent	CO <sub>2</sub> - combin- ing power.		Sp. gr.
Aug. 1.....	69.1	2,382	100.1	158.8	19.7	1,650	1.038	660	15.94	99.4	0.200	64	1.074	No alkali.
" 2.....	69.2	2,421	100.1	163.0	19.7	1,982	1.034	704	20.03	89.6				
" 3.....	69.4	2,351	100.1	154.4	19.7	1,838	1.035	597	19.04	88.2	0.183			
" 4.....	69.4	2,351	100.1	154.4	20.1	2,240	1.030	224	18.44	80.4				900 cc. Tyrode's so- lution + 3 per cent NaHCO <sub>3</sub> by duodenal tube.
" 5.....	70.9	2,351	100.1	154.4	20.1	2,012	1.030	91	16.62	73.9	0.149			
" 6.....	71.7	2,435	100.1	163.4	19.7	2,150	1.028	75	15.3	64.5				
" 7.....	71.9	2,435	100.1	163.4	19.7	1,395	1.033	230	13.7	57.0	0.165	86	1.066	No alkali.
" 8.....	71.4	2,435	100.1	163.4	20.1	1,520	1.032	433	15.8	65.5				
" 9.....	71.4	2,435	100.1	163.4	20.1	1,600	1.030	464	16.0	60.6	0.133		1.073	
" 10.....	71.6	2,435	100.1	163.4	20.1	1,518	1.031	471	15.1	52.8				
" 11.....	71.0	2,435	100.1	163.4	20.1	2,008	1.028	472	16.8	66.5	0.143		1.071	
" 12.....	71.4	2,435	100.1	163.4	20.1	2,135	1.024	406	15.9	59.5				500 cc. 0.3 per cent Na <sub>2</sub> CO <sub>3</sub> .
" 13.....	71.4	2,422	100.1	162.1	20.1	1,800	1.028	386	15.7	66.5	0.107	69		" 0.5 "
" 14.....	71.5	2,435	100.1	163.4	20.1	2,320	1.025	336	17.9	70.5				" 0.5 "

Aug. 15.....	71.2	2,435	100.1	163.4	20.1	1,830	1.026	403	16.6	64.5	0.159	79	1.076	No alkali. 500 cc. 1 per cent Na <sub>2</sub> CO <sub>3</sub> by duo- denal tube.
" 16.....	71.6	2,435	100.1	163.4	20.1	2,023	1.020	288	15.9	44.7				
" 17.....	71.4	2,435	100.1	163.4	20.1	1,862	1.027	372	17.9	55.2	0.165	79		No alkali.
" 18.....	71.5	2,435	100.1	163.4	20.1	1,885	1.024	443	18.2	50.9				
" 19.....	71.6	2,435	100.1	163.4	19.7	1,890	1.026		18.4	47.9	0.172	67	1.071	500 cc. 1 per cent Na <sub>2</sub> CO <sub>3</sub> by duo- denal tube.
" 20.....	70.9	2,435	100.1	163.4	19.7	1,455	1.027	356	17.2	35.8				No alkali.
" 21.....	71.1	2,435	100.1	163.4	19.7	1,865	1.024	252	18.5	34.6				500 cc. 1 per cent Na <sub>2</sub> CO <sub>3</sub> by duo- denal tube.
" 22.....	70.4	2,427	98.7	163.3	20.0	1,456	1.027	430	16.9	35.4				No alkali.
" 23.....	70.6	2,462	97.5	167.7	20.0	1,600	1.028	408	17.5	35.1	0.173	66	1.073	
" 24.....	70.8	1,467	95.3	61.9	20.6	1,835	1.033	459	20.5	29.9				
" 25.....	70.0					2,370	1.012			0.0				

The most encouraging fact with regard to this patient was the complete cure of his gangrenous toes. Several days before he left the hospital the lesions were completely healed and the redness had disappeared. Moreover, there was total absence of pain both in the feet and legs.

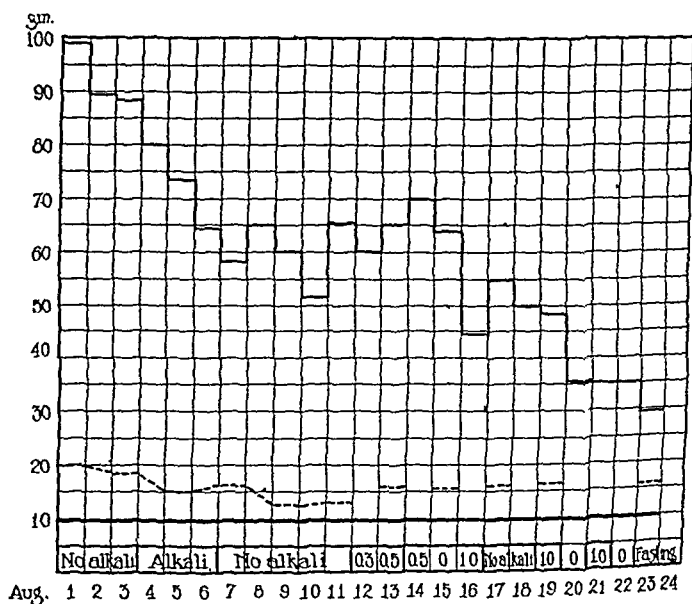


FIG. 3. Case 4. — Gm. of sugar in urine. --- Hundredths of a per cent blood sugar. 0.3, 0.5, 1.0 denote per cent of sodium carbonate.

**Case 5.**—B. B., a muscular, well nourished man, age about 20 years. Physical examination negative. Formerly drank considerable beer and some whiskey, but none of either for the past 4 years. Smokes fifteen to twenty cigarettes daily. Remembers no childhood diseases. Always well until 4 years ago, when he began to lose weight, became weak and very thirsty. Micturition ten to twelve times daily. Had frequent headaches. Had taken treatment in 1912, including 2 days' starvation. Urine became sugar-free and he gained 4 pounds. Kept at work till 7 months ago. Became careless regarding diet and grew worse, losing 8 pounds. For the past 3 weeks has been on "diabetic diet." Referred by Dr. Stillman of the Rockefeller Hospital.

This case came too late to afford us much opportunity for study. It is reported only for the result of the administration of

TABLE V.  
Case 5. B. B.

Date, day ending.	Food.	Urine.					Blood.		
		Volume.	Sp. gr.	Acidity 0.1 N.	N.	G.	Sugar.	CO <sub>2</sub> -com- bining power.	Sp. gr.
		cc.		cc.			per cent		
Aug. 25..	15 gm. carbohydrate and 35 calories per kg .....	995	1.034	547	18.99	26.7			
" 26..	1 sandwich (stolen).	810	1.029	490		12.8	0.213	69.1	1.073
" 27..	Fasting.....	680	1.023	398	12.32	0.0			
" 28..	500 cc. milk + 0.5 per cent Na <sub>2</sub> CO <sub>3</sub> by duodenal tube.	595	1.023	371	11.01	0.0	0.108	80.4*	1.072
" 29..	1,000 cc. milk + 0.5 per cent Na <sub>2</sub> CO <sub>3</sub> by duo- denal tube.....	650	1.026	552	13.48	0.0			
" 30..	1,000 cc. milk by stomach .....	495	1.027	401	11.67	2.0-			

\* Blood drawn before any alkali was given.

milk containing alkali by duodenal tube. After the urine had been rendered free of sugar by starvation, first 500 cc. and the next day 1 liter of milk were given after being boiled to guard against excessive putrefaction, and being rendered alkaline to the extent of 0.5 per cent of the anhydrous carbonate. The urine continued free of sugar, but the next day when 1,000 cc. of raw milk were given by stomach the resulting urine contained roughly 2 gm. of sugar. Does this signify that acid chyme brought out the sugar (4)?

The relationship between the blood sugar and CO<sub>2</sub>-combining power is also of interest.

#### *Analysis of Feces.*

To show that the infusion of alkali up to 1 per cent (anhydrous carbonate) directly into the intestine does not interfere with digestion, we show in Table VI the analyses of feces from three patients, carefully separated so as to correspond with periods during which alkali was given and periods when it was not



given. For Case 1 the analysis of feces also shows the comparative utilization of the foodstuffs with and without artificial digestion. Time did not suffice to make direct analyses for carbohydrate also, but for this patient the digestion of carbohydrate is clearly indicated by the amount of sugar in the urine before and after using lactopeptine and Merck's pancreatin<sup>7</sup> (Fig. 1).

TABLE VI.  
*Feces. Daily Averages.*

Nature of period.	Period date.	No of days.	N in food.	N in urine.	N in feces.	N balance.	Fat in food.	Fat in feces.	Fat balance.
<i>Case 1.</i>									
No alkali. 5 gm. lactopeptine before each meal.	Aug. 1-4	4	gm. 13.7	gm. 9.47	gm. 7.29	gm. -3.06	gm. 131.9		
No alkali. 10 gm. lactopeptine before each meal.	" 5-9	5	gm. 14.0	gm. 9.62	gm. 3.95	gm. +0.043	gm. 132.0	gm. 24.0	gm. +108.0
500 cc. 0.3 per cent $\text{Na}_2\text{CO}_3$ in duodenum. 10 gm. lactopeptine before each meal.	" 10-15	6	gm. 14.3	gm. 8.73	gm. 4.07	gm. +1.5	gm. 132.4	gm. 24.3	gm. +108.1
No alkali. No digestive powder.	" 16-19	4	gm. 14.3	gm. 6.75	gm. 10.10	gm. -2.55	gm. 132.4	gm. 85.03	gm. + 47.37
<i>Case 3.</i>									
500 cc. 1 per cent $\text{Na}_2\text{CO}_3$ in duodenum.....	" 7-11	5	gm. 17.6	gm. 17.9	gm. 1.17	gm. -1.47	gm. 171.1	gm. 2.25	
No alkali.....	" 12-15	4	gm. 17.6	gm. 18.8	gm. 1.45	gm. -2.65	gm. 171.1	gm. 3.47	
<i>Case 4.</i>									
No alkali.....	" 7-11	5	gm. 20.1	gm. 15.5	gm. 1.81	gm. +3.8	gm. 163.4		
0.3 to 1.0 per cent $\text{Na}_2\text{CO}_3$ in duodenum.....	" 12-16	5	gm. 20.3	gm. 16.4	gm. 1.45	gm. +3.45	gm. 163.4		

<sup>7</sup> No allowance has been made for the possible occurrence of lactose in these powders. Assuming 40 per cent, which would be high, the amount of starch digested would be some 12 gm. at most, less than the number of grams of sugar in the urine.

## DISCUSSION.

The foregoing results appear to confirm the observations made by Murlin and Kramer on the effects of alkali with depancrea-tized dogs. Owing probably to the more conservative use of the alkali the urine was not rendered entirely free of sugar, at least not for a whole day; but it was in several instances very materially reduced. The rise in excretion following the alkali (carbonate) leaves no doubt of this effect. Because of the preliminary character of these observations we did not seek to maintain an alkaline urine. The acidity figures in the tables show how nearly this state was approached. An alkaline urine on such a diet as was given here does not represent a normal condition of affairs, and we see no reason for the enormous doses of alkali which are often administered. In fact the results which have followed the use of sodium bicarbonate in these experiments (in agreement with the observations on dogs) indicate clearly that this salt may do real harm rather than good. From these brief experiences we are prepared to recommend the normal salt rather than bicarbonate, and are certain it can be used intraduodenally up to 1 per cent with entire safety. However, if it is kept up too long there is possibility of sloughing. Signs of this were seen in one case, although it was not serious.

A word is due regarding the gain in weight so often witnessed in patients treated with alkali. A good instance is seen in Case 2 of this series. This patient gained about 4 kg. during the time the alkali was being given, and held most of it for 4 or 5 days after the alkali was discontinued. Doubtless this increase represents water almost exclusively. Attempts have been made to explain the retention of glucose by alkali by supposing that it is held back in this water. Two objections can be offered to this: (1) the greatest retention of water rarely corresponds to the maximum retention (decreased elimination) of glucose; and (2) there is no reason to suppose that the fluid retained in the tissues would contain more sugar than the blood. If it did not contain more the total retention in this water would rarely account for more than a small fraction of the total retention (decreased elimination) of glucose.

The figures for blood sugar and specific gravity of the blood

prove, we think conclusively, that there may be a *bona fide* reduction in the percentage of blood sugar by the alkali independently of any factor of dilution. If this is true, there is little reason to doubt that the alkali may, in man as in the dog, facilitate the actual oxidation of glucose. Only direct observations on the respiratory metabolism can settle this question.

It should be noted that there was distinct clinical improvement in several of the patients during the course of the treatment. This was most marked in Cases 2 and 4. All felt an improvement, but it was not clearly objective except in these two.

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# THE RELATIONSHIP EXISTING BETWEEN THE OXIDASE ACTIVITY OF PLANT JUICES AND THEIR HYDROGEN ION CONCENTRATIONS, WITH A NOTE ON THE CAUSE OF OXIDASE ACTIVITY IN PLANT TISSUES.\*

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## INTRODUCTION.

It is peculiar to tissues of many plants to hasten greatly the oxidation by atmospheric oxygen of certain aromatic compounds of the hydroquinone type. This capacity of plant tissues, which differs in degree in different plants as well as with different aromatic compounds used, has been termed oxidase activity.

Some years ago a study was started in the Office of Plant Physiological and Fermentation Investigations in the Bureau of Plant Industry on the relationship of the oxidase activity to various physiological conditions of plants as well as to certain plant diseases. While no complete review of the first part of the work has yet been made, the second part of the work has yielded numerous results, parts of which have been published.<sup>1,2</sup> The rôle of hydrogen ions in various physiological processes has been reviewed by McClendon,<sup>3</sup> and their significance in enzymotic proc-

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<sup>1</sup> True, R. H., Physiological observations on alkaloids, latex, and oxidases in *Papaver somniferum*, *Am. J. Bot.*, 1916, iii, 1.

<sup>2</sup> Bunzell, H. H., The measurement of the oxidase content of plant juices, *U. S. Dept. Agric., Bureau of Plant Industry, Bull.* 238, 1912; A biochemical study of the curly-top sugar beets, *ibid.*, *Bull.* 277, 1913; Oxidases in healthy and in curly-dwarf potatoes, *J. Agric. Research*, 1914, ii, 373.

<sup>3</sup> McClendon, J. F., Hydrogen and hydroxyl ion concentration in physiology and medicine, *Med. Review of Reviews*, 1916, xxii, 333.

esses by Sørensen.<sup>4</sup> It has been known for some time that the color reactions assigned to the presence of oxidases are interfered with by small quantities of acids, alkalies, and poisons.<sup>5</sup>

The writer undertook to study the oxidase activities of various plant tissues in different hydrogen ion concentrations. It was felt that such a study would not only throw some light on the nature of the "acid effect," but would also furnish us with further data on the comparative behavior of the oxidases from different sources.

### *Methods.*

(a) *Preparation of Plant Material.*—Immediately after collection the plant tissues were placed in a vacuum desiccator and dried over lime. By frequently exhausting the desiccators it was possible to effect fairly rapid drying. The dried material was finely ground and passed through a 100 mesh sieve. When, after repeated sifting and regrinding of the remainder, about 90 per cent of the material had passed through the sieve, the two parts were intimately mixed. The samples were preserved in desiccators over lime.

(b) *Determination of Oxidase Activity.*—These determinations were made with the simplified apparatus<sup>6</sup> in the usual way.<sup>7</sup> There were no facilities available at this time for the determination in the constant temperature room of the laboratory. During the summer months it was practically impossible to maintain the temperature constant within one of the small boxes below 35°C. For this reason the measurements of the oxidase activity were carried out at room temperature and the temperature was recorded to 0.1°C. at the time of each reading. The final readings were corrected at the beginning of the experiment, according to the following formula:

$$R = 76 - \frac{(76 - R_1) T}{T_1}$$

<sup>4</sup> Sorensen, S. P. L., *Enzymstudien*. II, *Biochem. Z.*, 1909, xxi, 131, 201; 1909, xxii, 352.

<sup>5</sup> For literature see Oppenheimer, C., *Die Fermente*, Leipzig, 4th edition, 1913, ii, 796.

<sup>6</sup> Bunzell, A simplified and inexpensive oxidase apparatus, *J. Biol. Chem.*, 1914, xvii, 409.

In the formula  $R_1$  and  $T_1$  are the observed final reading and the temperature of the box at the time of reading in degrees absolute temperature, while  $R$  and  $T$  are the corrected reading and the temperature of the box in degrees absolute temperature at the beginning of the experiment.

(c) *Hydrogen Ion Determinations.*—The hydrogen ion determinations were made according to the gas chain method.

A Wolff potentiometer was used with a Siemens and Halske galvanometer. The latter produced on application of 1 microvolt to the terminals a deflection of 4 mm. on a scale about 1.5 m. distant from the mirror. A Weston standard cell was used, calibrated at the beginning of the determinations by the makers and at the end by the National Bureau of Standards. The potentiometer readings were made to 0.0001 volt at 5 minute intervals, and the values considered final when two successive readings agreed to 0.0002 volt. In most cases they agreed to 0.0001 volt. A 0.1 N calomel electrode was used in these experiments which was at times compared with another 0.1 N calomel electrode, but more frequently with the potential of a hydrogen electrode immersed in a standard acetate solution.<sup>7</sup> In the first comparison the agreement was to 0.0001 volt; in the second, the values for  $P_H$  were from 4.618 to 4.631 at different times during the period of experimentation. The liquid connection was made through an ungreased stopcock as recommended by Clark,<sup>8</sup> and a vessel containing 0.1 N KCl was interposed between the 0.1 N calomel electrode and the vessel containing the saturated KCl solution. Two bent tubes filled with 0.1 N KCl established connection between the calomel electrode and the intermediate vessel on the one hand and the intermediate vessel and the saturated KCl solution on the other.

After introduction of the liquid into the hydrogen-filled vessel, the latter was shaken at the rate of sixty-eight excursions per minute. The shaking machine designed in the laboratory<sup>9</sup> proved ideal for this purpose. The shaking was continued for 10 minutes, then the shaking machine was stopped, all stopcocks were opened except the ungreased stopcock connecting the saturated KCl solution with the hydrogen electrode vessel, and a reading was made.

(d) *Maintenance of Constant Temperature.*—For all the experiments described in this paper, as well as for similar experiments in the future, a small room 11 feet long, 5½ feet wide, and 7½ feet high was constructed.

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<sup>7</sup> Walpole, G. S., Hydrogen potentials of mixtures of acetic acid and sodium acetate, *J. Chem. Soc.*, 1914, cv, 2501.

<sup>8</sup> Clark, W. M., A new hydrogen electrode vessel, *J. Biol. Chem.*, 1915, xxiii, 475.

<sup>9</sup> Bunzell, U. S. Dept. Agric., Bureau of Plant Industry, *Bull.* 238, 1912, 17.

It is fairly well insulated, provided with a double glass window on one end and an entrance through a small antechamber on the other. Within this room there is another separate compartment as long as the room itself, to which access can be had by means of four glass doors. The temperature in the main chamber in which the experimenter works is constant within a few tenths of a degree; that in the separate compartment, within one-tenth. The temperature of the main chamber is about  $0.5^{\circ}\text{C}.$  below that of the special compartment, so that when one of the glass doors is opened temporarily no marked change in temperature takes place in the smaller compartment. There is a separate cooling coil running the length of the ceiling of the main room as well as of the smaller compartment. Water is circulated through these coils to cool the air below the temperature desired.

In summer the water is cooled in a specially constructed ice box built into the constant temperature room. The rate of circulation is regulated by means of valves at the outflow. By means of fans the cooled air is blown over heating wires and lamps, and the current through these automatically regulated by means of relays and thermoregulators. Hasselbring's modification of the Bunnell and Hasselbring<sup>10</sup> thermoregulator is used. The temperature of the box is set for a temperature of  $25\text{--}26^{\circ}\text{C}.$

(e) *General.*—The general plan of experimentation was as follows: The oxidase activity of the particular plant material toward the reagent (pyrocatechol was chosen for all of these experiments) was determined at different concentrations of hydrogen ions. If the data obtained were not of such a nature as to give a satisfactory curve, additional experiments were made wherever possible. Even so, the data are not sufficiently numerous to give the curves any real value except at the points of intersection with the abscissas.

Each oxidase determination was made in duplicate and the mean values are given in all cases. The various concentrations of hydrogen ions desired could be fairly closely approximated by means of acetic acid—sodium acetate mixtures,  $0.1\text{ N}$  acetic acid,  $0.01\text{ N}$  acetic acid, and  $0.01\text{ N}$   $\text{HCl}$ . 4 cc. of the solutions fixing the hydrogen ion concentration were used with 2 cc. of a 0.5 per cent pyrocatechol solution. The plant material was chosen in such quantities as to give a final reading of about 3 cm. of mercury. The plant material with the acid solutions was placed in the larger compartment of the oxidase apparatus, and the pyro-

<sup>10</sup> Bunnell, H. H., and Hasselbring, H., A thermo-regulator for electrically regulated constant temperature chambers, *J. Am. Chem. Soc.*, 1914, xxxvi, 249.

catechol solution in the smaller. It was essential to work swiftly in order to eliminate appreciable deterioration of the plant material in the acid solutions. For the determinations of the hydrogen ion concentrations, four times the quantity of the materials used in the oxidase measurements were taken. The solutions proper were allowed to stand in the constant temperature room for about  $\frac{1}{2}$  to 1 hour to assume the temperature of the latter, and the plant powder was added just prior to the introduction of the mixture into the electrode vessel. In this way only the initial hydrogen ion concentrations were determined. When the mixture examined consisted merely of plant material, pyrocatechol, and water, no definite value was obtained at once and the potential drifted toward an approximate (slightly fluctuating) end-point. In these cases it was assumed that the  $C_H$  of the solution changed during the measurement. To get at the original values, curves were plotted with the times on one axis and the potential difference values on the other, and the potential difference existing at the beginning was obtained by interpolation.

#### EXPERIMENTAL.

0.01 gm. of the pyrocatechol was used in all these experiments in the form of 2 cc. of a 0.5 per cent solution. The solutions used for obtaining the desired hydrogen ion concentrations were used in quantities of 4 cc.

To be able to compare the oxidase activity of a certain plant material at a fixed hydrogen ion concentration, with its activity at a different hydrogen ion concentration, it was necessary to choose some standard of comparison. The activity in aqueous solutions containing merely pyrocatechol and the plant material was chosen as such a standard. Moreover, since it is unnecessarily laborious to adjust the quantities of plant material in such a way as to obtain the same activity in such nearly neutral solutions with all samples of the same type of plant material, the following procedure was adopted. The imaginary quantity of plant material chosen for each series was such that in the absence of acid mixtures a maximal absorption of about 3 cm. was produced. On the basis of previous work<sup>11</sup> together with experi-

<sup>11</sup> Bunzell, The mode of action of the oxidases, *J. Biol. Chem.*, 1916, xxiv, 91.



TABLE I.

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	C <sub>H</sub>
Series I. Initial temperature of the constant temperature box, 26°C.; final, 26.2°.					
0.10 gm. of fine powder of dried potato peel.	18 N $\text{HC}_2\text{H}_3\text{O}_2$ 2 N NaOH	0	0	3.661*	$2.18 \times 10^{-4}$
"	15 N $\text{HC}_2\text{H}_3\text{O}_2$ 10 N NaOH	1.05	1.10	4.895*	$1.27 \times 10^{-4}$
"	8.5 N $\text{HC}_2\text{H}_3\text{O}_2$ 8 N NaOH	1.60	1.65	5.851*	$1.41 \times 10^{-4}$
"	9 N $\text{H}_3\text{PO}_4$ 15 N NaOH	2.07	2.12	6.902	$1.25 \times 10^{-7}$

Series II. Temperature throughout, 24.9°.

.01 gm. of fine powder of liriodendron buds, with two coats of scales removed.	N $\text{HC}_2\text{H}_3\text{O}_2$	0	0	2.428	$3.73 \times 10^{-3}$
"	0.5 N $\text{HC}_2\text{H}_3\text{O}_2$	0.12	0.12	2.631	$2.34 \times 10^{-4}$
"	0.1 N $\text{HC}_2\text{H}_3\text{O}_2$	0.87	0.87	3.088	$8.17 \times 10^{-4}$
"	$\text{H}_2\text{O}$	2.85	2.85	5.897†	$1.26 \times 10^{-4}$

Series III. Initial temperature, 26°; final, 26.2°.

0.02 gm. of fine powder of dried liriodendron bud scales.	N $\text{HC}_2\text{H}_3\text{O}_2$	0.60	0.65	2.428	$3.73 \times 10^{-3}$
"	0.5 N $\text{HC}_2\text{H}_3\text{O}_2$	0.60	0.65	2.724	$1.89 \times 10^{-3}$
"	0.1 N $\text{HC}_2\text{H}_3\text{O}_2$	0.80	0.85	3.211	$6.15 \times 10^{-4}$
"	$\text{H}_2\text{O}$	3.27	3.32	5.897	$1.26 \times 10^{-4}$

\* These values are copied from Series VI, since there was not sufficient plant material available for the determinations.

† Copied from Series III.

TABLE I—Continued.

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	C <sub>H</sub>
Series IV. Initial temperature, 24.5°; final, 24°.					
0.02 gm. of fine powder of dried liriodendron bud scales.	cc. 0.1 N HCl	0	0	1.203	6.27x10 <sup>-2</sup>
"	N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.45	0.32	2.521	3.01x10 <sup>-3</sup>
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.37	2.25	4.912	1.22x10 <sup>-3</sup>
"	4 N NaOH	2.80	2.68	5.897	1.26x10 <sup>-6</sup>
"	H <sub>2</sub> O				
Series V. Initial temperature, 23°; final, 23.1°.					
0.02 gm. of fine powder of dried liriodendron bud scales.	0.05 N HCl	0	0	1.431	3.71x10 <sup>-2</sup>
"	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.62	1.65	3.692	2.03x10 <sup>-4</sup>
"	1 N NaOH	3.17	3.19	5.954	1.01x10 <sup>-3</sup>
"	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	3.40	3.42	5.897	1.26x10 <sup>-6</sup>
"	8 N NaOH				
"	H <sub>2</sub> O				
Series VI.* Temperature throughout, 25°.					
0.05 gm. of the powdered potato material which passed through the sieve.	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0	0	3.661	2.18x10 <sup>-4</sup>
"	1 N NaOH				
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.80	0.80	4.895	1.27x10 <sup>-3</sup>
"	4 N NaOH	1.42	1.42	5.851	1.41x10 <sup>-4</sup>
"	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.65	2.65	6.176	6.67x10 <sup>-7</sup>
"	8 N NaOH				
"	H <sub>2</sub> O				

\* The material used for Series VI was prepared as follows: Market potatoes were washed and wiped dry; they were then peeled and the peelings dried in *vacuo* over lime. After grinding in a small mill and sifting through a 100 mesh sieve, most of the material passed through. On microscopic examination this proved to be mainly starch; the residue was mainly cortex. The two portions were examined separately. In this series the finer powder (mostly starch) was examined. The microscopic examination was made by Dr. H. Hasselbring, and my thanks are extended to him herewith.

TABLE 1—Continued.

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	C <sub>H</sub>
Series VII.* Initial temperature, 24°; final, 24.1°.					
0.05 gm. of the pow- dered potato ma- terial which did not pass through the sieve.	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.05	0.05	3.570	2.69x10 <sup>-4</sup>
	1 N NaOH				
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.25	2.27	4.875	1.33x10 <sup>-3</sup>
	4 N NaOH				
"	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	3.05	3.07	5.827	1.49x10 <sup>-4</sup>
	8 N NaOH				
"	H <sub>2</sub> O	3.95	3.97	5.905	1.24x10 <sup>-4</sup>

Series VIII.† Initial temperature, 24°; in Experiments 3 and 4 the final temperature was 24.3°, and in Experiments 5, 6, 7, and 8 it was 24.8°.

0.05 gm. of fine pow- der of Tulip A.	N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0	0	2.822	1.51x10 <sup>-3</sup>
"	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.35	0.43	5.969	1.07x10 <sup>-4</sup>
	1 N NaOH				
"	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.22	2.42	5.939	1.115x10 <sup>-4</sup>
	8 N NaOH				
"	H <sub>2</sub> O	2.55	2.75	5.939	1.115x10 <sup>-4</sup>

Series IX. Initial temperature, 26.2°; in Experiments 1 and 2 the final temperature was 26.4°, in Experiments 3 and 4, 26.7°, and in Experiments 5, 6, 7, and 8, 27°.

0.05 gm. of fine pow- der of Tulip A.	0.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.35	0.40		
"	10 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.75	0.88	4.013†	9.70x10 <sup>-3</sup>
	2 N NaOH				
"	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.27	2.47	5.939	1.15x10 <sup>-4</sup>
	8 N NaOH				
"	H <sub>2</sub> O	2.65	2.85	5.939	1.15x10 <sup>-4</sup>

\* In Series VII the material used was that which, after thorough grinding in a mill, did not pass through the 100 mesh sieve.

† Tulip tree leaves were collected from a tree at Arlington in June, 1915. This sample will be referred to as Tulip A in distinction to Tulip B, denoting leaves collected from the same tree in June, 1916. (From this tree all samples of tulip tree material used in these investigations were collected.)

‡ Copied from Series X.

TABLE I.—Continued.

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	C <sub>H</sub>
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Series X. Initial temperature, 28.3°; in Experiments 1 and 2 the final temperature was 28.5°, and in Experiments 3, 4, 5, 6, 7, and 8 it was 28.8°.

0.05 gm. of fine powder of Tulip B.*	cc. N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.12	0.17	2.838	1.45x10 <sup>-3</sup>
"	10 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.95	1.07	4.013	9.70x10 <sup>-5</sup>
"	2 N NaOH				
"	5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	3.15	3.27	5.218	6.05x10 <sup>-6</sup>
"	4 N NaOH				
"	H <sub>2</sub> O	3.65	3.77	5.939	1.15x10 <sup>-6</sup>

Series XI.† Initial and final temperatures in Experiments 1 and 2, 24.1°; in Experiments 3 and 4 the final temperature was 24.4°, and in Experiments 5, 6, 7, and 8 it was 24.8°.

0.03 gm. of fine powder of dried potato sprouts.	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.12	0.12	3.736	1.84x10 <sup>-4</sup>
"	1 N NaOH				
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.42	1.50	4.915	1.22x10 <sup>-5</sup>
"	4 N NaOH				
"	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.82	1.99	5.926	1.19x10 <sup>-6</sup>
"	8 N NaOH				
"	H <sub>2</sub> O	2.20	2.37	5.922	1.20x10 <sup>-6</sup>

Series XII.‡ Initial temperature, 25.1°; final temperature, 25.5° in all of the experiments.

0.05 gm. of fine powder of dried magnolia leaves.	9 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.70	0.80		
"	1 N NaOH				
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.55	1.65	4.834§	1.31x10 <sup>-5</sup>
"	4 N NaOH				
"	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.32	2.42	5.710	1.95x10 <sup>-5</sup>
"	H <sub>2</sub> O	2.20	2.30		

\* For further description of this material see note to Series VIII.

† Potatoes bought in the Fall of 1915 were allowed to sprout in the laboratory. The sprouts were removed and dried in a desiccator *in vacuo* over lime. Some of the mother tubers were cut into thin slices and treated in the same way. The material obtained from the mother tubers proved to be inactive and will not be referred to further in this paper.

‡ Magnolia leaves and flower petals were collected from a tree on the Department grounds in June, 1915.

§ On account of lack of material this determination had to be made with 1916 magnolia material. This seems justified by the fact that nearly equal results are obtained under such circumstances. For instance, where 1915 leaves with 0.10 HC<sub>2</sub>H<sub>3</sub>O<sub>2</sub> gave a P<sub>H</sub> of 3.382, 1916 leaves gave 3.453 under identical conditions.

TABLE I—Continued.

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	C <sub>H</sub>
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Series XIII. Initial temperature, 25.8°; final, 25.9° in all of the experiments.

0.05 gm. of fine powder of dried magnolia leaves.	cc. N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0	0	2.632	2.33x10 <sup>-3</sup>
"	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.55	0.58	3.382	4.15x10 <sup>-4</sup>

Series XIV. Initial temperature, 24.5°; in Experiments 1, 2, 3, and 4 the final temperature was 24.2°, and in Experiments 5, 6, 7, and 8, 24.1°.

0.02 gm. of fine powder of dried magnolia flower petals collected simultaneously with leaves (Series XII and XIII).	N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.10	0.02	2.567	2.71x10 <sup>-3</sup>
"	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.40	0.32	3.314	4.85x10 <sup>-4</sup>
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.67	1.57	4.926	1.19x10 <sup>-3</sup>
"	4 N NaOH } H <sub>2</sub> O	2.32	2.22	5.263*	5.46x10 <sup>-4</sup>

Series XV.† Initial temperature, 26.5°; in Experiments 1, 2, 3, and 4 the final temperature was 27°, and in Experiments 5, 6, 7, and 8, 27.5°.

0.02 gm. of fine powder of dried magnolia leaves (1916).	N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.15	0.28	2.497	3.19x10 <sup>-3</sup>
"	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.17	1.29	3.186	6.52x10 <sup>-4</sup>
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	2.47	2.61	4.884	1.31x10 <sup>-3</sup>
"	4 N NaOH } H <sub>2</sub> O	3.40	3.64	5.398	4.00x10 <sup>-4</sup>

\* See second note to Series XII.

† Magnolia leaves, flower petals, and stamens were collected June 15, 1916, from the same tree which furnished material for the foregoing series.

TABLE I—*Concluded.*

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	C <sub>H</sub>
Series XVI. Initial temperature, 27.7°; in Experiments 3 and 4 the final temperature was 23.1°, and in Experiments 5, 6, 7, and 8, 23.2°.					
0.01 gm. of fine powder of dried magnolia flower petals (1916).	cc. N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0	0	2.489	3.24x10 <sup>-3</sup>
"	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.45	0.55	3.298	5.04x10 <sup>-4</sup>
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.20	1.32	4.892	1.28x10 <sup>-5</sup>
"	4 N NaOH				
"	H <sub>2</sub> O	2.55	2.67	5.263	5.46x10 <sup>-6</sup>

Series XVII. Initial temperature, 27.2°; in Experiments 1, 2, 3, and 4 the final temperature was 27.2°, and in Experiments 5, 6, 7, and 8, 28.0°.

0.01 gm. of dried stamens from magnolia flowers (1916).	N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0	0	2.508	3.10x10 <sup>-3</sup>
"	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0	0	3.083	8.26x10 <sup>-4</sup>
"	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.35	1.55	4.890	1.29x10 <sup>-5</sup>
"	4 N NaOH				
"	H <sub>2</sub> O	1.70	1.90	5.545	2.86x10 <sup>-6</sup>

ments described in Series XVIII and XIX, it was possible to calculate what the maximal oxygen absorption would be in all experiments of a series if the quantities of plant material used in each experiment would give an absorption of exactly 3 cm. in a neutral solution.

The great advantage of this method of comparison will be evident later on when the comparative activity of the same material in different hydrogen ion concentrations will be represented by curves.

To test whether the maximal absorption is directly proportional to the quantity of plant material used in distinctly acid solutions, as was found to be the case in neutral and nearly neutral solutions, the following experiments were tried.

TABLE II.

Material.	Solution.	Final read- ing.	Cor- rected read- ing.	P <sub>H</sub>	G <sub>H</sub>
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Series XVIII. Initial temperature, 24.8°; final temperature in all of the experiments, 24.7°.

0.02 gm. of fine powder of dried magnolia flower petals (1915).	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	0.75	0.72	3.314	4.85x10 <sup>-4</sup>
0.03 "	0.1 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.10	1.08	(3.314)*	(4.85x10 <sup>-4</sup> )
0.02 "	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.50	1.48	4.926	1.19x10 <sup>-3</sup>
0.03 "	4 N NaOH	2.50	2.48	(4.926)*	(1.19x10 <sup>-3</sup> )
	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>				
	4 N NaOH				

Series XIX. Initial temperature, 27.2°; in Experiments 1, 2, 3, 4, 7, and 8 the final temperature was 27.9°, and in Experiments 5 and 6, 28°.

0.05 gm. of fine sifted powder of potato peel (Series VI).	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	1.12	1.29	4.895	1.27x10 <sup>-3</sup>
0.10 "	4 N NaOH	2.50	2.67	(4.985)*	(1.27x10 <sup>-3</sup> )
0.10 "	6 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	3.45	3.64	5.851	1.41x10 <sup>-4</sup>
0.05 "	4 N NaOH	1.62	1.79	(5.851)*	(1.41x10 <sup>-4</sup> )
	8.5 N HC <sub>2</sub> H <sub>3</sub> O <sub>2</sub>				
	8 N NaOH				

\* No separate experiments seemed necessary to determine the hydrogen ion concentrations in these mixtures.

Experiments in Series XVIII and XIX prove that in acid solutions also the oxidase activity is approximately proportional to the quantity of material used.

The results were then recalculated on the basis that the absorption in the assumedly acid-free mixture was 3 cm. By means of direct proportionality the other values of the same series were arrived at. The results obtained in this way are summarized, as follows:

TABLE III.

Material.	Oxidase activity.	P <sub>H</sub>
Potato peel powder, 1915.....	0	3.661
“.....	1.56	4.895
“.....	2.34	5.851
“.....	3.00	6.902
Potato peel powder, 1916 (passed through sieve)....	0	3.661
“.....	0.91	4.895
“.....	1.61	5.851
“.....	3.00	6.176
Potato peel powder, 1916 (not passed through sieve)	0.04	3.570
“.....	1.72	4.875
“.....	2.33	5.827
“.....	3.00	5.905
Potato sprouts.....	0.15	3.736
“.....	1.90	4.915
“.....	2.52	5.926
“.....	3.00	5.922
Scaled tulip tree buds, 1916.....	0	2.428
“.....	0.13	2.631
“.....	0.92	3.088
“.....	3.00	5.897
“.....	0.59	2.428
“.....	0.59	2.724
“.....	0.77	3.211
“.....	3.00	5.897
“.....	0	1.203
“.....	0.36	2.521
“.....	2.52	4.912
“.....	3.00	5.897
“.....	0	1.431
“.....	1.45	3.692
“.....	2.80	5.954
“.....	3.00	5.897
Tulip tree leaves, 1915.....	0	2.822
“.....	0.47	
“.....	2.64	5.969
“.....	3.00	5.939
“.....	0.42	
“.....	0.93	4.013
“.....	2.60	
“.....	3.00	5.939
Tulip tree leaves, 1916.....	0.14	2.838
“.....	0.85	4.013
“.....	2.60	5.218
“.....	3.00	5.939



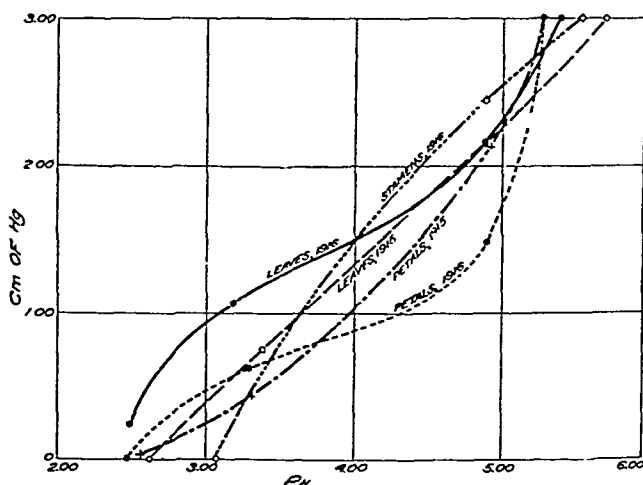


FIG. 3. Curves showing the relationship existing between  $P_H$  and oxidase activity of different magnolia material.

other aromatic compounds acting as good oxidase reagents are oxidized by atmospheric oxygen at a measurable speed in weakly alkaline solutions; it would therefore be impossible, with the present stage of technique, to differentiate between the effect due to the alkalinity of the solution and that due to the oxidase activity of the plant material.

The experiments are not sufficient in number to establish clearly the quantitative relationships existing between the extent of the oxidase activity of a plant material and the concentration of hydrogen ions existing in the medium. When this relationship is worked out it will be found no doubt to consist of at least two factors; one the direct destructive effect of the acidity on the active matter, the other the retardation of the rate of oxidation by the hydrogen ions. It should not be difficult to separate these two factors. A study of the behavior of different reagents under identical conditions will also throw light on the question of the identity of the factors underlying the biological oxidation of different oxidase reagents.

The experiments already made seem to bring out an interesting point, which, if thoroughly corroborated by further evidence, would prove important from the plant physiological point of view. If Figs. 1, 2, and 3 are examined, it appears that the hydrogen

ion concentrations corresponding to complete inhibition cover a rather narrow range for each type of plant materials; *i.e.*, for potato, no matter what kind of potato material was used, all inhibiting hydrogen ion concentrations range from 3.55 to 3.70. For the tulip tree material, whether scaled buds, scales, or leaves were used, the range lies very much higher, between 2.30 and 2.80. In the case of magnolia the range is 2.45 to 3.05, not much different from the tulip tree. This seems significant. It would indicate that the sensitiveness to acidity of all the oxidase factors throughout the same plant is approximately uniform; moreover, since different specimens of the same genus, collected at different times of the year, were used, it would indicate also that the acid sensitiveness figure is a rather fixed number, characteristic for any particular genus. It would even seem that the acid sensitiveness constant is the same or nearly the same for different genera (tulip tree and magnolia) of the same family (Magnoliaceæ).

It would seem, therefore, that the nature of the material which is responsible for the phenomena of oxidase activity is identical in closely related plants. It has already been suggested in a previous paper<sup>12</sup> that the oxidase activities exhibited by certain plant tissues are due to the presence of a certain class of colloids of a chemical nature or physiochemical state peculiar to the particular genus or family of plants in which they occur. If the observations recorded in this paper are fully corroborated by future experiments, we should have a strong support for such a hypothesis.<sup>13</sup>

On account of the wide occurrence of oxidase activity among plants, it is to be expected that some widely occurring constituent lies at the bottom of it. At the present time we do not know what that is. From what we know about the mode of oxidase activity, this constituent is easily destroyed by acids, by chemical means such as shaking, by heat, or by alcohol; it gradually changes to an inactive form on standing in aqueous solutions, but will keep practically indefinitely when dry. Such a constituent would have to be of rather universal distribution in plants and would have to occur in many different modifications in order to result in the

<sup>12</sup> Bunzell, The relative oxidase activity of different organs of the same plant, *J. Biol. Chem.*, 1916, xxiv, 103. See also Traube.<sup>15</sup>

<sup>13</sup> The effect of acids on colloids is clearly discussed by W. W. Taylor, *The Chemistry of Colloids*, London, 1915.



# A HITHERTO NEGLECTED FACTOR AFFECTING THE DETERMINATION OF MINUTE QUANTITIES OF CREATININE.

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The determination of blood creatinine, whether according to the original technique of Folin<sup>1</sup> or to some modification identical in principle,<sup>2</sup> has become so much a matter of clinical routine, and has been so generally accepted as a valuable laboratory aid to diagnosis and prognosis, that it is, to say the least, discouraging to learn that the method "gives no information whatever concerning the amount of creatinine present" in the blood. This, nevertheless, is the conclusion reached in a recent paper by McCrudden and Sargent.<sup>3</sup> The essence of their argument is found in the assertion that in an average blood analysis as much as five-sixths of the color upon which the determination is based may be due merely to sodium picrate; and the assertion is supported by what at first sight appears to be sufficiently convincing evidence. Fortunately it is possible to show that the evidence may be in itself a correct statement of facts observed, and the conclusion none the less totally unjustified.

Like McCrudden and Sargent, we encountered recently, in a series of blood creatinine determinations, a number of irregular and indeed incredible results, which for a time seemed to us irreconcilable with any claim of accuracy that might be made for the method. Since, however, our earlier results had been both reasonable and consistent, it seemed to us, on consideration,

<sup>1</sup> Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

<sup>2</sup> Such as that described by Myers, V. C., and Fine, M. S., *Chemical Composition of the Blood in Health and Disease*, Cooperstown, N. Y., 1915, p. 19.

<sup>3</sup> McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxvi, 527

that the unacceptable character of the later ones must be due not to an intrinsic worthlessness of the method, but to some unrecognized disturbing factor associated somehow with the lapse of time. An investigation of the circumstances revealed the correctness of this suspicion and restored our confidence in the method; and, as we believe that McCrudden and Sargent's difficulties are to be explained in the same manner as our own, and may possibly have been encountered by others, we think it proper to publish now an account of our experience.

It may be stated at once that the cause of our anomalous results was found to lie in the circumstance, not previously noted or at least not previously commented on, that the successful determination of minute quantities of creatinine is possible only when all the picric acid solutions employed are reasonably *fresh*. In what follows the essential character of this condition will be amply illustrated, and it will be shown that, while failure to observe it would account for the results of McCrudden, and Sargent, a due attention to its fulfillment confers on the method a highly satisfactory degree of accuracy.

#### *The Color Reaction of Fresh Picric Acid Solutions with Sodium Hydroxide.*

When a freshly prepared picric acid solution is treated with sodium hydroxide, the color of the mixture deepens regularly as the amount of the alkali is increased. This fact, which was recognized by Folin<sup>1</sup> and which indeed cannot have escaped the attention of anyone who has attempted to check the method, constitutes the reason for insisting upon the use in creatinine determinations of a constant and accurately measured quantity of sodium hydroxide. If, now, the proportions of the two reagents are those prescribed in work with blood, *i.e.*, 0.5 cc. of 10 per cent NaOH to 10 cc. of saturated picric acid, the color of the mixture, which is now a slightly alkaline solution of sodium picrate, is hardly to the unaided eye appreciably different from that of the picric acid solution itself. It does not rival in intensity that developed in a standard solution containing as little as 0.02 mg. of creatinine in 10 cc. of saturated picric acid. Careful comparison in the colorimeter does indeed show the picrate

solution to be distinctly deeper in hue than the picric acid; but it is still, by actual measurement, two and a half times less highly colored than the very dilute creatinine solution mentioned. Figures justifying these statements will be found among the data of Table I, to be referred to presently. They are obviously irreconcilable with the contention of McCrudden and Sargent, that the color of sodium picrate itself is so intense as to overshadow completely the Jaffé reaction in an average specimen of blood.<sup>4</sup>

*The Color Reaction of Old Picric Acid Solutions with Sodium Hydroxide.*

Solutions of picric acid which have stood for any length of time in the laboratory exhibit a behavior very different from that of fresh solutions.<sup>5</sup> Apparently they always contain more or less of some substance which gives with sodium hydroxide, in the regulation amount, a color reaction not only identical with that of creatinine, but on occasion so powerful that it would entirely mask the simultaneous presence of the latter. The development of this chromogenic substance is illustrated by the behavior of the following series of variously aged picric acid solutions.

<sup>4</sup> McCrudden and Sargent calculated that, gram for gram, creatinine gives a color only 500 times as intense as picrate. Their argument starts from the observation that "a solution containing 0.12 gm. of picric acid and 0.25 mg. of creatinine in 10 cc. gives approximately twice as deep a color as a solution containing 0.12 gm. of picrate alone in 10 cc." An inspection of our Table III will show that when the picric acid is freshly dissolved, the amount of creatinine which will just about double the color of 10 cc. of saturated picric acid is only 0.015 mg. Starting from this basis it would follow that, gram for gram, creatinine gives  $\frac{120}{0.015} = 8,000$  times as much color as picrate.

<sup>5</sup> The picric acid which we employ is furnished by one of the best known American manufacturers. The only specimen in our possession having possibly a different source is the one mentioned in the text as being over 2 years old; this had probably been imported from Germany. Strictly speaking, our observations apply in the meantime only to these two brands of the reagent; it is, of course, possible that they are to be accounted for by some impurity from which other samples might be free.

1. A saturated solution, 18 days old, which had been exposed since its preparation to the diffuse light of the laboratory.

2. A saturated solution of similar history, but 1 month old.

3. A 1 per cent solution, 2 months old, which had been kept in the dark since its preparation.

4. A portion of the previous solution which had been exposed during 1 month (Aug. 20 to Sept. 20) to the practically continuous sunshine of the daylight hours.

5. A saturated solution, known to be approximately 6 months old, kept in a rather brightly illuminated room, but not subject to the action of direct sunlight.

6. A saturated solution, regarding which it could be stated with certainty only that it was at least 2 years old; it has stood on a window shelf exposed to all the sunlight of the morning hours.

7. A fresh saturated solution of the purified picric acid obtained from Solution 6 by evaporation and double recrystallization.

To each of these solutions there was added 10 per cent sodium hydroxide in the proportion of 5 cc. per 100, and after an interval of 10 minutes they were compared one by one with a *fresh* picric acid solution of similar concentration (saturated or 1 per cent as the case might be) which had been likewise treated with the specified amount of alkali. This we shall refer to as the sodium picrate standard. The standard was set each time at a depth convenient for the particular comparison in hand. The data obtained are shown in Table I. There are included in this table, as justification for certain statements in the preceding section of the paper, colorimetric comparisons, with the sodium picrate standard, of two additional solutions—a freshly made solution containing 0.2 mg. of creatinine in 100 cc. of saturated picric acid treated with the proper amount of alkali (Solution 8), and a fresh picric acid without any addition of sodium hydroxide (Solution 9). The unit employed in calculating the relative depth of color is always the color of the standard sodium picrate of equivalent concentration. The last column of the table is an attempt to describe roughly the intensity of the reaction as it appeared to the unaided eye. Each figure of the fifth column is an average representing from three to five experiments.

We do not claim for the figures of Table I more than an approximate accuracy. There are several reasons for this. One is that with the lighter shades of yellow very close duplication of readings is difficult of attainment. The darker solutions, on the other hand, do not, even in thin layers, exactly match the tint of the sodium picrate, and do not within 10 minutes reach their maximum intensity. The figures do nevertheless give some kind of numerical expression to the striking differences perceptible to the eye, and reveal also the slighter degrees of color change which the eye might fail to detect. It will be seen at a glance that the older a picric acid solution is, the deeper color it yields upon treatment with sodium hydroxide. Even within 18 days the effect of exposure in solution is perceptible. Solution 5, a month old, would be obviously useless for the

TABLE I.

*Comparison of Picric Acid Solutions of Different Age and History.*

Solution No.	Age of solution.	Character of light to which it was exposed.	Standard set at.	Reading of solution.	Relative depth of color. (Sodium picrate = 1.)	Appearance as compared with untreated picric acid.
			mm.	mm.		
1	18 days.	Diffuse.	20	16.6	1.2	Very slight change.
2	1 mo.	"	30	22.3	1.3	"
3	2 "	Dark.	30	31.6	0.95	Hardly any change.
4	1 "	Sunlight.	30	3.8*	8	Striking red.
5	6 "	Diffuse.	50	<5*	>10	" "
6	2 yrs.	Intermittent sunlight.	50	<1*	>50	Extremely deep brownish red.
7**	Fresh.		50	30	1.7	Slight deepening.
8†	"		20	8*	2.5	Very distinct deepening.
9‡	"		20	37	0.54	

\* Colors not strictly comparable.

\*\* Recrystallized from No. 6.

† Containing 0.2 mg. of creatinine per 100 cc.

‡ Not treated with alkali.

purposes of a blood creatinine determination; while the oldest of all gave a reaction of astonishing intensity.

A point of considerable interest becomes apparent on a comparison of Nos. 4 and 5. These were portions of the same solution, of which the first had been kept for 2 months in the dark, and the second exposed to all the sunlight available during a month of brilliant and cloudless weather. Upon the addition of alkali the first did not become any darker than a similarly treated fresh solution; while the second developed a rich red, comparable to that of a normal solution of potassium bichromate. Evidently light plays an important and possibly essential part in the chemical change which leads to the appearance of the chromogenic material. This material, whatever its nature, can be removed by ordinary processes of purification. This is shown by the behavior of Solution 7, which contained a picric acid prepared by double recrystallization from the highly contaminated Solution 6. It had evidently been almost, though not entirely, freed from the reacting impurity.

The color developed in the older solutions reminds one of sodium picramate; and, in fact, in order to match exactly the color of an old picric acid solution treated with alkali, it is sufficient to add picramic acid to the standard with which it is compared. Such a composite standard was made by dissolving 10 mg. of picramic acid in 100 cc. of fresh saturated picric acid. Solutions 4, 5, and 6 were then read against this standard,



the last (No. 6) being first diluted four times with fresh picric acid solution, and all (including the standard) being treated with the proper amount of sodium hydroxide. The results were the following, a perfect match being obtained in every case.

Solution 4.	Standard at 15 mm;	reading	9.6
" 5.	" " 20 "	; "	10.0
" 6.	" " 50 "	; "	15.6

From these data, if we suppose for a moment that the reacting substance might be picramic acid, it may be calculated that there were present in 100 cc. of Solution 4, 16 mg.; of Solution 5, 20 mg.; and of Solution 6, no less than 128 mg. These figures give a much more accurate idea of the relative intensity of the reaction in these three solutions than the data of Table I. When it is remembered that Solution 4 was only 1 per cent, while No. 5 was saturated (1.2 per cent), it will be seen that in reality 1 month's exposure to sunlight had as much effect as 6 months' exposure to diffuse daylight.

It is clear from the foregoing that if we were to use such a picric acid solution as No. 4 to precipitate the blood proteins, a subsequent attempt to determine creatinine in the filtrate would, if the standard were correct, yield an impossibly high result; while, if the standard also were made up with the same picric acid, the results would be of just the kind reported by McCrudden and Sargent; *i.e.*, slight differences in an already low creatinine content would hardly affect the colorimetric values at all.

#### *The Deterioration with Age of Standard Creatinine Solutions in Picric Acid.*

This leads us to remark that naturally the solutions of creatinine in picric acid which form the usual standards in blood work are likewise subject to increase of colorimetric value through age. This is well illustrated in Table II. Three standard solutions were found in the laboratory which had been prepared 5 months previously, and which, while securely stoppered, had been in no way protected from light. They contained respectively 0.2, 0.5, and 1.0 mg. of creatinine in 100 cc. For comparison with these we prepared from a fresh picric acid solution three other standards of the same concentrations. All were treated according to the usual technique, and compared with the fresh 0.5 mg. standard set at 20 mm. The results appear in Table II.

Obviously the old standards are quite useless. It is perhaps superfluous to point out again that the results which they yield, when compared with each other, are precisely of the same character as those of McCrudden and Sargent.

TABLE II.

*Standard: 0.5 Mg. of Creatinine per 100 Cc., Set at 20 Mm.*

Creatinine per 100 cc.	Age.	Actual reading.	Theoretical reading.
mg.		mm.	mm.
0.2	Fresh.	43.5	50
0.2	Old.	9.7	
0.5	Fresh.	20.2	20
0.5	Old.	6.5	
1.0	Fresh.	10.5	10
1.0	Old.	5.8	

*The Actual Accuracy of the Folin Method for Determination of Creatinine in the Blood.*

We have brought forward evidence sufficient to show that one cannot anticipate even approximately correct results in the determination of blood creatinine, unless every picric acid solution employed, whether for the precipitation of the proteins or in the preparation of the standards, is at least reasonably fresh. It remains to ascertain whether, when that condition is fulfilled, the results are actually to be depended upon. The only way to do so is to imitate the technique for blood by comparing with a selected standard each one of a series of fresh picric acid solutions to which known amounts of creatinine have been added. We have accordingly simply repeated the first experiment of McCruden and Sargent, using, however, a more extended series of test solutions, and taking the single additional precaution upon the necessity of which we have insisted.

With the aid of a micro burette graduated in fiftieths, such as is used in Bang's method for the determination of blood sugar, there were measured out 0.2, 0.4, . . . up to 2 cc. of a 0.005 per cent solution of creatinine in picric acid, and these volumes were then made up to 20 cc. with saturated picric acid; the series was continued by taking 1.1, 1.2 . . . up to 2 cc., and diluting to 10. In this way were prepared twenty solutions containing from 0.05 to 1.00 mg. of creatinine per 100 cc., with steps of 0.05 mg. To each was added one-twentieth volume of 10 per cent NaOH. The solution containing 0.5 mg. creatinine per 100 cc. was taken as the standard. After the lapse of 10 minutes each solution was compared in the Duboscq colorimeter with the standard at 20 mm.

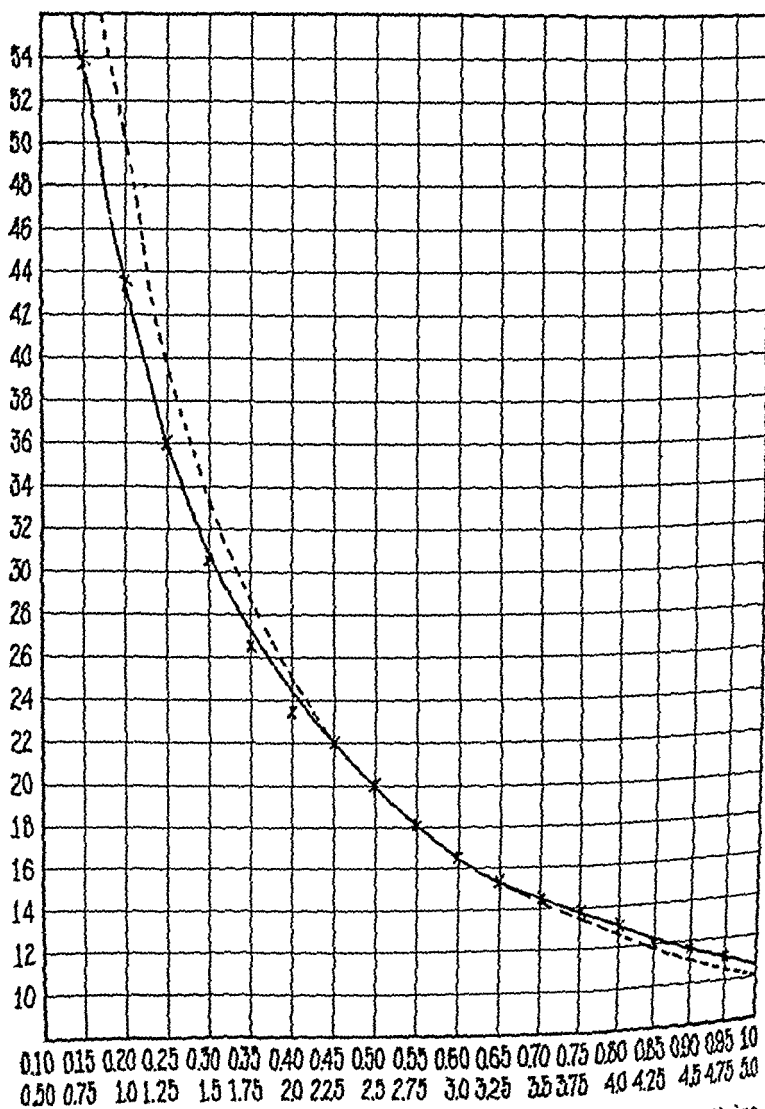


CHART 1. For use with a standard containing 0.5 mg. of creatinine per 100 cc., set at 20 mm. The ordinates are colorimeter readings, the actual position of which is marked by crosses. The abscissae indicate mg. of creatinine per 100 cc. (upper row of figures); the lower figures give the amount of blood creatinine corresponding. The readings calculated on a basis of inverse proportionality are shown by the dotted line.

The entire experiment was carried out twice. The results are recorded in Table III, and graphically illustrated in Chart I. In the latter, space is saved by omitting the extreme left hand end of the curve, which possesses little practical value.

TABLE III.

*Readings, Actual and Calculated, against a Standard Containing 0.5 Mg. of Creatinine per 100 Cc.*

Creatinine solution *	Creatinine per 100 cc	Colorimeter readings			
		Series 1	Series 2	Average	Calculated
<i>cc</i>	<i>mg</i>				
0 0	0 00	100**		100	∞
0 1	0 05	80**	94**	87	200
0 2	0 10	63**	64**	63 5	100
0 3	0 15	53	55	54	66 6
0 4	0 20	44	43	43 5	50 0
0 5	0 25	36 0	36 1	36 1	40 0
0 6	0 30	30 6	30 6	30 6	33 3
0 7	0 35	26 6	26 5	26 6	28 6
0 8	0 40	23 6	23 6	23 6	25 0
0 9	0 45	21 7	21 8	21 8	22 2
1 0	0 50	20 0	20 0	20 0	20 0
1 1	0 55	17 7	17 9	17 8	18 2
1 2	0 60	16 6	16 3	16 5	16 7
1 3	0 65	15 3	15 3	15 3	15 4
1 4	0 70	14 0	14 3	14 2	14 3
1 5	0 75	13 7	13 7	13 7	13 3
1 6	0 80	12 9	12 8	12 9	12 5
1 7	0 85	12 1	11 9	12 0	11 8
1 8	0 90	11 7	11 4	11 6	11 1
1 9	0 95	11 0	11 1	11 1	10 5
2 0	1 00	10 5	10 5	10 5	10 0

\* Referred to a total volume of 10 cc.

\*\* Standard at 10 mm Actual reading multiplied by 2.

Since McCrudden and Sargent employed the same standard that we did, our figures and chart, over the region duplicated, are directly comparable with theirs. The character of the results is seen at a glance to be entirely different. It is readily understood by a reference to the chart. The average readings are there seen to lie without exception very close to a smooth curve. This curve in its middle part coincides almost exactly

with the calculated one, and deviates notably from the latter only for the smaller quantities of creatinine. The assumption of strict inverse proportionality between creatinine content and colorimetric readings would involve an error not exceeding 5 to 10 per cent between 0.30 and 0.40 mg. per 100 cc., or 3 to 5 per cent between 0.75 and 1.00, and in the intervening region one almost negligible. For smaller amounts than 0.30 mg., the percentage error rapidly increases. Here the influence of the constant sodium picrate color becomes, in relation to the diminishing creatinine effect, increasingly prominent. This influence is far from being so overwhelming as in the curve of McCrudden and Sargent, but of course it does exist.

If the curve is plotted upon a larger scale on coordinate paper, it affords a means, at once more convenient and more accurate than calculation, of ascertaining the true content of creatinine corresponding to a given colorimeter reading. If it is constructed from the user's own observations it helps to correct any systematic personal bias in the reading of the colorimeter. It also enables one to a considerable extent to dispense with a multiplicity of standards. With the aid of Chart 1, for instance, it is possible, using a single standard solution of 0.5 mg. per 100 cc., to cover successfully the whole range of possible creatinine values between 0.15 and 1.00 mg. per 100, corresponding to blood creatinine contents of 0.75 to 5.00 mg. per 100 cc.<sup>6</sup>

The curve can, of course, by using all the figures of the table, be further prolonged to the left. Certain practical difficulties, however, detract from the usefulness of such an extension. A reading of 55 mm. is at the limit of the usual colorimeter's capacity; moreover, when the colored solution to be read is very much feebler than the standard, its tint does not exactly match, and it becomes almost impossible to obtain successive readings within reasonable range of each other; lastly, even if the first difficulty is largely overcome by setting the standard at 10 mm. instead of 20 mm., the volume of filtrate required is greater than that usually available in the ordinary routine.

<sup>6</sup> The idea of constructing such a curve in order to escape the necessity of using a standard within certain limits only has already been utilized by Mellanby, E., *J. Physiol.*, 1907-08, xxxvi, 447.

For these reasons we consider it advisable, if the blood creatinine is low, and especially if it falls below 1.0 mg. per 100 cc., to adopt a standard of 0.2 mg. per 100 cc., and we have constructed a curve (Chart 2) available for use with the same. This was done from the data of Table IV, which exhibits the readings given by the first ten creatinine solutions of Table III, when these were compared with the standard mentioned. The standard was again set at 20 mm. As before, two series of observations were made; but in this instance one set was allowed after addition of

TABLE IV.

*Readings, Actual and Calculated, against a Standard Containing 0.2 Mg. of Creatinine per 100 Cc.*

Creatinine solution.*	Creatinine per 100 cc.	Colorimeter readings.			
		Series 1.	Series 2.	Average.	Calculated.
<i>cc.</i>	<i>mg.</i>				
0.1	0.05	43	40	42	80.0
0.2	0.10	32	32	32	40.0
0.3	0.15	24.2	23.6	23.9	26.7
0.4	0.20	20.0	20.0	20.0	20.0
0.5	0.25	17.2	17.0	17.1	16.0
0.6	0.30	15.3	15.3	15.3	13.3
0.7	0.35	13.0		13.0	11.4
0.8	0.40	11.8	12.0	11.9	10.0
0.9	0.45	10.5	10.7	10.6	8.9
1.0	0.50	9.4	9.7	9.6	8.0

\* Referred to a total volume of 10 cc.

the alkali, to stand for 24 hours before the readings were taken. It will be seen that this made no appreciable difference to the result. The 24 hour standard, when compared with a fresh one, proved also to be identical in depth of color. These faintly colored solutions are therefore remarkably stable, and the statement of Folin<sup>7</sup> that "it is possible to so make the reaction that the color does not fade in the course of 24 hours," a statement made in connection with the technique for urinary creatinine, is found to be equally applicable to the case of blood.

Table IV and Chart 2 are perhaps of special interest in that the standard here employed is the one recommended by Folin

<sup>7</sup> Folin, *J. Biol. Chem.*, 1914, xvii, 469.

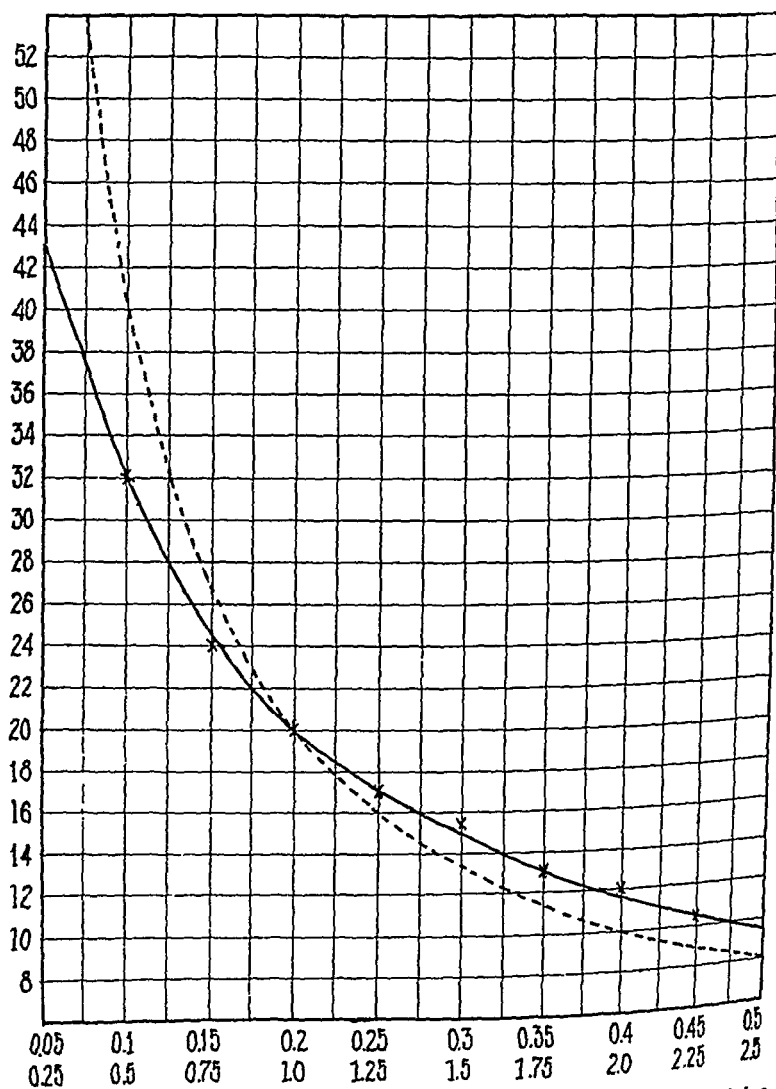


CHART 2. For use with a standard containing 0.2 mg. of creatinine per 100 cc., set at 20 mm. The interpretation is the same as with Chart 1.

for use with the average sample of normal blood. According to Folin<sup>1,7</sup> the readings obey the law of inverse proportionality so long as neither the standard nor the unknown is more than one and a half times as strong as the other. This would allow here a range of 0.133 to 0.30 mg., corresponding to 0.66 to 1.50 mg. per 100 cc. of blood. Our observations show that within this range the relation does indeed hold fairly well, the assumption of its rigid exactness not being likely to involve an error greater than about 10 per cent. Obviously, however, the use of the curve on Chart 2 not only makes one independent of the correctness or otherwise of the rule, but extends greatly the region within which the standard in question is applicable. Results in which one may have reasonable confidence can be read off the chart all the way between 0.05 and 0.5 mg., corresponding to a blood content of 0.25 to 2.5 mg. per 100 cc. The error inherent in the calculation, whenever standard and unknown are widely different, is thus avoided, and the only serious source of error remaining is in the reading of the colorimeter.

As for the general character of Curve 2, there is no question but that it deviates, on either side of the reference point, more rapidly from the calculated than does Curve 1; nor can it be doubted, we think, that the picrate color, here thrown into greater relative prominence, is responsible for this outcome. Since, however, the deviations are regular, and the results reproducible, the picture presented is still far from affording a justification for the utter condemnation of the method. On the contrary, by showing the exact nature and extent of the departure from theory, it puts the method on an even firmer basis than before.<sup>8</sup>

#### SUMMARY AND CONCLUSIONS.

The action of light, with possibly other agencies, upon picric acid solutions results in the development of some substance giving a red reaction with sodium hydroxide. The presence of

<sup>8</sup> We have not had occasion to test the applicability of Folin's latest method for the determination of creatinine in urine; but if the evidence adduced in support of the technique for blood has any weight at all, it will apply even more strongly to the case of urine, where the quantities dealt with are considerably larger, and the concentration of sodium picrate is less.



this substance renders old solutions unsuitable for use in the determination of minute quantities of creatinine. In the determination of blood creatinine all picric acid solutions employed should therefore be less than a month old, or should have been kept since their preparation in the dark. They should in any case be tested before use for the presence of the chromogenic material. These recommendations apply not only to simple picric acid solutions, but equally to the solutions of creatinine in picric acid which serve as standards. Indeed, for the precipitation of blood proteins it would be simplest to avoid all danger by following the technique of Myers and Fine,<sup>2</sup> and using only dry picric acid. Picric acid in solution need then be employed only in making up standards. The length of time during which solutions protected from light remain safe has not yet been ascertained; but it certainly exceeds 2 months.

When the precautions mentioned are attended to, the Folin method for creatinine determination in the blood possesses a high grade of accuracy. Its accuracy as well as its convenience is increased by the use of suitably prepared curves showing the actual relation of color intensity to creatinine concentration over a wider range than is generally considered permissible with a single standard. Two such curves are here presented, covering between them a range of 0.25 to 5 mg. of creatinine per 100 cc. of blood.

# IMPURE PICRIC ACID AS A SOURCE OF ERROR IN CREATINE AND CREATININE DETERMINATIONS.

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the Massachusetts General Hospital, Boston.)

(Received for publication, November 8, 1916.)

In the course of the last few months McCrudden and Sargent have published two critical papers on the colorimetric method for the determination of creatine and creatinine. Their first paper<sup>1</sup> describes some seemingly simple yet convincing experiments indicating that normal human urine contains some substance ("x-creatine") which gives the creatinine reaction when boiled with picric acid as in Folin's micro method<sup>2</sup> for the determination of creatine, yet gives no such reaction after heating with hydrochloric acid (as in the older macro method). The second paper<sup>3</sup> has to do with the influence of the color from the sodium picrate in the determination of creatinine in blood and urine. The last two paragraphs of this paper are as follows:

"From the data it is clear that in the determination of creatinine in the blood the color due to creatinine is such a small proportion of the total color that analysis gives no information whatever concerning the amount of creatinine present; the slight variations obtained in duplicates can be accounted for by slight variations in the amount of picric acid in the solution.

*In the light of these experiments it is clear that all that has been written hitherto concerning the physiology of creatinine and creatine needs careful revision; much of it will have to be modified, some of it—all that concerning creatinine and creatine in the blood, for example—will have to be rejected altogether."*

We have repeated the work of McCrudden and Sargent with reference to the determination of creatine in urine and of creati-

<sup>1</sup> McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxiv, 423.

<sup>2</sup> Folin, O., *J. Biol. Chem.*, 1914, xvii, 472, 479.

<sup>3</sup> McCrudden and Sargent, *J. Biol. Chem.*, 1916, xxvi, 527.

nine in blood, and in both instances have obtained results which are entirely different from the results reported by them.

Table I, containing some calculated and experimental figures taken from the second paper of McCrudden and Sargent and also the corresponding experimental figures obtained by ourselves, indicates the magnitude of the discrepancy between their data and ours.

TABLE I.

*A Comparison of Calculated Colorimetric Readings with Those Obtained (a) by McCrudden and Sargent and (b) by Ourselves.*

No.	a.	b.	Calculated.
	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
1	25.5	26.6	28.6
2	21.7	24.8	25.0
3	23.4	22.3	22.2
4	20.0	20.0	20.0
5	21.2	18.2	18.2
6	18.7	16.7	16.7
7	20.4	15.7	15.4
8	18.0	14.3	14.3

The differences between our results and those of McCrudden and Sargent manifestly lie far outside any reasonable limits of experimental error, and cannot be explained on the basis of some slight variation in the conditions under which the two sets of determinations were made. In the course of our endeavor to find an adequate explanation we examined the different samples of picric acid in our laboratory with reference to the color of their sodium picrates in terms of creatinine. McCrudden and Sargent had made such a comparison and had found that creatinine, weight for weight, gives about 500 times as deep a color with picric acid and alkali as is given by the picric acid and alkali alone. In all we examined six different samples of picric acid, of which four had been bought in dry form and the fifth in the more recent wet condition in which picric acid is now almost always sold; the sixth was a product obtained from the fifth by recrystallization of the sodium salt, according to directions given below.

According to our observations creatinine gave about 3,000 times as deep a color as was given by our poorest picric acid (the wet sample), when alkali was added exactly as in the creatinine determination. Using our best, the recrystallized picric acid, creatinine gave about 12,000 times as deep a color as its sodium picrate.

In view of the remarkably faint color of our sodium picrates in comparison with the color recorded by McCrudden and Sargent, it occurred to us that the endeavor of American manufacturers to meet the enormous demands, created by the war, for picric acid, may have resulted in the production of extremely low grades of picric acid, and that McCrudden and Sargent may have been using an unusually impure sample.

We therefore communicated with Dr. McCrudden, and, with him, tested his picric acid and compared it with ours. The results obtained were quite astounding, and it became at once clear to us, as well as to Dr. McCrudden, that the observations published by McCrudden and Sargent had been obtained on the basis of an extraordinarily impure picric acid. The results were so different from the results obtained with any sample of our picric acid that the conclusions formulated by McCrudden and Sargent have no bearing whatever on the results obtained with approximately pure picric acid.

It remains to be said, however, that the picric acid used by McCrudden and Sargent was labelled c.p., and it is scarcely to be supposed that other investigators may not have bought and used for creatinine determinations picric acid of similar low grade. We agree with McCrudden and Sargent that any creatinine or creatine determinations made on blood by the help of such impure picric acid would be worthless.

From our examination of a number of different samples of wet picric acid we have obtained the impression that with the introduction of this "safe" mode of shipping, the manufacturers must have introduced some unsatisfactory change in the process of preparing the product. We have not yet seen a single sample of wet picric acid as pure as any of the four dry samples in our possession.

As a simple test of the purity of picric acid for use in connection with creatinine determinations we propose the following.

To 20 cc. of a saturated (1.2 per cent) solution of picric acid add 1 cc. of 10 per cent sodium hydroxide and let it stand for 15 minutes. The color of the alkaline picrate solution thus obtained must not be more than about twice as deep as the color of the saturated acid solution. If the quality of the picric acid is good, the color of the picrate solution will be no deeper at the end of 24 hours than at the end of 15 minutes provided that organic impurities, dust, etc., be excluded. If the picric acid is unusually pure, the color of the picrate solution will not be more than one and a half times as deep as that of a saturated picric acid solution; *i.e.*, by setting the picric acid solution at 20 mm. in the Duboscq colorimeter, the picrate will give a reading of 13 to 14 mm.

To purify picric acid proceed as follows: Transfer about 600 gm. of wet picric acid, or about a pound of dry picric acid, to a large beaker (capacity not less than 4 liters). Pour on boiling water until the beaker is nearly full and add 200 cc. of saturated (50 per cent) sodium hydroxide solution. Stir, and if necessary heat again until all the picric acid has dissolved, yielding a deep red picrate solution. To the hot solution add rather slowly, with stirring, 200 gm. of sodium chloride. Cool in running water to about 30°C., with occasional stirring. Filter on a large Buchner funnel and wash a few times with 5 per cent sodium chloride solution. Transfer the picrate to the large beaker, fill with boiling water, and when the picrate is dissolved add, with stirring, first 50 cc. of 10 per cent sodium hydroxide solution, and then 100 gm. of sodium chloride. Cool to 30°C., with stirring, filter, and wash with sodium chloride solution, as before. Repeat the solution and precipitation of the sodium picrate twice more, but for the last washing of the last precipitated picrate use distilled water instead of sodium chloride solution.

Dissolve the purified picrate in the same large beaker, with boiling distilled water, and filter hot on a large folded filter, collecting the filtrate in a large flask. To the hot filtrate add 100 cc. of concentrated sulfuric acid, previously diluted with about two volumes of water. The liberated picric acid begins to come out at once. Put a beaker over the mouth of the flask and cool under running tap water to about 30°C. Filter with suction as before and wash free from sulfates with distilled water.

While the findings of McCrudden and Sargent are abundantly accounted for on the basis of impurities in their picric acid, it of course remains a fact that the yellow color of pure sodium picrate does theoretically interfere with the accuracy of the colorimetric creatinine estimations and practically does limit the dilu-

tion in which creatinine may be quantitatively determined. The creatinine solution obtained from normal blood after the proteins have been precipitated by the addition of four volumes of picric acid solution is strong enough in preformed creatinine to permit its estimation with a fair degree of accuracy.

A simple modification of the original procedure, which most investigators will probably accept as an improvement, is the following.

To the fresh blood add four volumes of saturated picric acid solution and about 1 gm. of powdered picric acid for each 10 cc. of blood used. Shake for 10 minutes, and filter. To 10 (or 20) cc. of the filtrate add 1 (or 2) cc. of a solution containing 7 per cent potassium hydroxide and 25 per cent potassium chloride. Let stand for 10 minutes, centrifuge, and compare in the colorimeter with the standard creatinine in picric acid to which has been added a corresponding amount of alkaline potassium solution. The potassium in the alkali precipitates fully 75 per cent of the picric acid present in a saturated solution, and thereby makes the color due to the minute amounts of creatinine present in normal blood distinctly more predominant than when the filtrates remain saturated solutions of picric acid.

In the above modification, as well as in the original method, the blood is diluted fivefold by the addition of four volumes of picric acid solution. The chief reason for this great dilution of the blood filtrates is that it enables one to make a creatinine determination with as little as 2 cc. of blood. When there is no reason for economy of blood, the creatinine concentration of the blood filtrates can advantageously be increased by using only two volumes of picric acid solution (+ solid picric acid) for the precipitation of the proteins. The precipitation in this case requires more time, about an hour. The standard creatinine solution in this case should, of course, be less dilute than when four volumes of picric acid are used for the precipitation.

Dr. McCrudden agreed with us that since we had been unable to find "x-creatine" in urine, this finding also is probably to be explained on the basis of impurities in the picric acid used by McCrudden and Sargent. To prove the point we determined the creatinine-creatine in three samples of urine, (a) by the old

method, (b) by the new (picric acid) method using our picric acid, and (c) by the new method using McCrudden's picric acid. The following values (in gm. per liter) were obtained.

TABLE II.

No.	a.	b.	c.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1	1.63	1.64	2.09
2	1.33	1.35	1.67
3	1.71	1.73	2.23

The figures recorded in Table II prove conclusively that the highly impure picric acid used by McCrudden and Sargent was responsible for their finding creatine in urines which did not contain any creatine.

Before communicating with Dr. McCrudden we had made a long series of determinations of creatinine and creatine in human urine. In this series we had determined the creatine-creatinine both by the old process and by Folin's micro method. The figures obtained are not without interest, because (1) they prove that the two methods give identical results, and (2) they are numerous enough to give a good idea as to the frequency with which creatine occurs in human urine, in health and in disease. The figures are given in the form of colorimetric readings only.

In explanation of these figures (Table III) it should be stated that in determining the creatine-creatinine we used as the standard the creatinine of the unheated urine (and not a standard creatinine solution).

TABLE III.

No.	Subject.	Diet.	Colorimetric readings.		
			Creatinine 1 mg. standard set at 20 mm.	Creatine-creatinine. Creatinine as standard set at 20 mm.	
				New method.	Old method.

*Normal Men.*

1	Normal.....	Mixed.		17.3	17.4
2	" .....	"		18.5	18.5
3	" .....	Creatine-free.	16.6	20.0	20.1
4	" .....	"	13.2	20.0	20.1
5	" .....	"	12.3	20.6	19.8
6	" .....	"	19.3	20.1	20.1
7	" .....	"	7.0	20.1	20.1
8	" .....	"	12.9	20.1	20.0
9	" .....	"	15.5	19.8	19.9
10	" .....	"	22.1	20.0	19.9
11	" .....	"	14.1	20.0	19.9
12	" .....	"	25.7	20.1	20.2
13	" .....	"	20.0	20.2	19.9
14	" .....	"	21.3	20.2	20.1
15	" .....	"	19.1	20.2	20.1
16	" .....	"	16.8	19.8	20.1
17	" .....	"	17.8	19.9	20.1
18	" .....	"	12.1	20.1	19.9
19	" .....	"	14.9	19.9	20.2
20	" .....	"	12.0	20.0	20.2
21	" .....	"	12.5	20.0	20.2
22	" .....	"	19.7	19.9	20.5
23	" .....	"	12.7	18.9	18.7
24	" .....	"	8.6	18.2	18.1
25	" .....	"	13.2	20.0	19.9
26	" .....	"	12.7	19.8	20.1
27	" .....	"	21.4	20.0	20.2

*Pathological Men.*

28	Diarrhea.....	Creatine-free.	16.6	18.3	18.1
29	Nephritis.....	"	15.5	17.0	16.9
30	Leukemia.....	Mixed.	16.2	14.7	14.9
31	Cardiac.....	"	26.9	10.8	11.0
32	" .....	"	13.0	18.0	18.0
33	Valvular heart.....	"	15.0	17.9	17.7
34	Mitral stenosis.....	"	24.8	16.7	17.1
35	Lung process.....	"	22.8	17.9	17.7
36	" .....	"	18.2	18.3	18.3



TABLE III—*Concluded.*

No.	Subject.	Diet.	Colorimetric readings.		
			Creatinine 1 mg. standard set at 20 mm.	Creatine-creatinine. Creatinine as standard set at 20 mm.	
				New method.	Old method.
Pathological Men.					
37	Tabes.....	Mixed.	15.1	18.7	19.1
38	Cirrhosis of liver.....	"	12.0	17.9	18.3
39	Renal anasarca.....	"	16.1	18.8	18.9
40	Pleurisy.....	"	15.0	16.8	16.6
41	" .....	"	19.1	17.8	18.0
42	" .....	"	17.8	17.6	17.8
43	" .....	"	13.3	17.2	17.2
44	" .....	"	16.2	14.5	14.8
45	" .....	"	19.6	8.8	5.2*
Pathological Women.					
46	Enteric.....	Mixed.	12.5	15.8	15.7
47	" .....	"	16.3	17.4	17.4
48	" .....	"	14.0	14.4	14.4
49	Gastric ulcer.....	Creatine-free.	17.6	14.8	15.1
50	Pneumonia.....	"	27.9	16.6	16.6
51	" .....	"	14.5	12.0	12.2
52	" .....	"	19.0	11.0	10.3
53	" .....	"	17.3	9.9	10.1
54	" .....	"	18.0	16.1	16.0
55	" .....	"	15.8	17.2	17.2
56	" .....	"	17.6	16.0	16.1
Pathological Children.					
57	Surgical.....	Mixed.	22.5	10.7	10.7
58	" .....	"	27.0	10.2	9.8
59	" .....	"	27.6	12.3	12.2
60	Syphilis.....	"	19.6	16.9	16.6
61	" .....	"	18.6	13.9	14.2
62	Eczema.....	Creatine-free.	20.4	14.1	14.2
63	" .....	"	16.4	10.6	10.4
64	" .....	"	14.5	17.9	18.2
65	" .....	"	27.1	17.1	17.1

\* Sugar.

# THE ESTIMATION OF AMINO-ACID NITROGEN IN BLOOD.

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(Received for publication, November 3, 1916.)

In spite of the rapidly growing literature upon the occurrence of amino-acids in blood and tissue under various conditions, little attention appears to have been given to a study of the comparative merits of various procedures for the preliminary removal of protein and manipulation of the solution prior to the analysis.

For the most part the original method of Van Slyke and Meyer<sup>1</sup> has been followed, in which the blood proteins are removed by precipitation with alcohol, the filtrate evaporated *in vacuo* to a small volume, and this solution used for analysis by the nitrous acid method.<sup>2</sup> Recent investigations by Folin and Denis<sup>3</sup> and particularly by Greenwald<sup>4</sup> have indicated that amino-acids do not completely escape precipitation by alcohol.

It was therefore considered desirable, as preliminary to some contemplated work upon the physiological occurrence of amino-acids, to compare the results obtained by the Van Slyke method, where different procedures were employed for the removal of the protein. It is the purpose of the present paper to report the results obtained in this connection, together with certain other findings which may be of interest.

Folin and Denis<sup>3</sup> state that certain nitrogenous substances added to the blood cannot be recovered quantitatively after precipitation with methyl alcohol.

Greenwald,<sup>4</sup> as a result of his study of this question, concluded that alcohol precipitates some nitrogenous non-protein con-

<sup>1</sup> Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

<sup>2</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

<sup>3</sup> Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

<sup>4</sup> Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

stituents of the blood, of which 25 to 50 per cent represents amino-acid nitrogen, as determined by Van Slyke's method. As it was originally planned in the present writer's work to substitute the Greenwald procedure of blood precipitation with trichloroacetic acid for the alcohol precipitation, a somewhat detailed study of the two methods was made.

TABLE I.

*Comparison between Alcohol Precipitation and Trichloroacetic Acid Precipitation of Blood. Non-Protein Nitrogen per 100 Cc. of Blood.*

Material.	Methyl alcohol precipitation.	Trichloroacetic acid precipitation.	Remarks.
	mg.	mg.	
Sheep blood (oxalated).....	28.70	37.60	By micro Kjeldahl. Distillates Nesslerized.
" " " .....	25.40	34.70	
Ox " (defibrinated).....	23.25	26.25	
" " " .....	22.12	25.90	
" " " .....	22.02	23.30	
" " " .....	18.31	24.30	
Calf " (oxalated).....	26.30	29.55	By micro Kjeldahl. Distillates titrated.
Ox " (defibrinated).....	21.10	25.60	
Ox blood (defibrinated).....	27.23	30.02	
" " " .....	30.57	31.82	
Sheep " " .....	28.20	27.37	
" " " .....	28.29	30.01	
Ox " + amino-acids.....	35.57	39.77	
" " (defibrinated).....	28.59	28.40	
Sheep " (oxalated).....	34.98	39.53	
" " " .....	35.64	36.75	
Pig blood (oxalated).....	22.93	23.88	By Kjeldahl.
" " " .....	29.92	32.03	
" " " .....	28.15	30.81	
Sheep " " .....	35.76	39.71	
" " " .....	35.48	38.40	

Determinations of the total non-protein nitrogen were made first. Samples of blood were precipitated according to Folin and Denis<sup>3</sup> and Greenwald.<sup>4</sup> Aliquot parts of the filtrates were digested and distilled as described in a previous paper.<sup>5</sup> The results are summarized in Table I. The figures given for Nessler-

<sup>3</sup> Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 47.

zation represent equivalents of 1 cc. of blood, those for micro distillation and titration with methyl red as indicator represent 3 to 5 cc. of blood. The last part of Table I gives Kjeldahl determinations on amounts of filtrates representing 30 to 40 cc. of blood. The micro determinations represent averages of three to seven determinations. The Kjeldahl figures represent averages of two to three determinations each.

The results show a decided loss of non-protein nitrogen where alcohol was used as precipitant. The differences are largest where Nesslerization was employed. This may be due, at least in part, to the small quantity of blood used in these determinations, whereby the limit of error is correspondingly increased. This may explain the much greater differences obtained by Greenwald when compared with the results obtained by titration in the present writer's results.

The precipitation of blood for the determination of amino-acid nitrogen was then tried. As urea and ammonia react somewhat with nitrous acid in the Van Slyke procedure, it was necessary to remove these constituents beforehand. A measured volume of blood (30 to 50 cc.) was introduced into a flask which contained 0.3 gm. of ground soy bean, a little water (2 to 3 cc.), and 1 cc. of a 3 per cent solution of  $\text{NaH}_2\text{PO}_4$ , and the mixture was gently agitated. After standing for  $\frac{1}{2}$  hour at room temperature, the mixture was precipitated by diluting to ten times its original volume with a 2.5 per cent solution of trichloroacetic acid. After standing for 30 minutes, it was filtered, the filtrate shaken with kaolin, and filtered again. An aliquot part of the filtrate was taken. The difficulty which presented itself at this stage was the removal of the trichloroacetic acid. The neutralization before removal is not practical, because the filtrate has to be evaporated to a very small volume and even fairly small amounts of salts give considerable trouble. The vacuum distillation, as in the original Van Slyke and Meyer method, was tried. The filtrate was evaporated at reduced pressure in a water bath. The usual arrangement of two distilling flasks was used and the reduced pressure obtained by means of a good water pump. The filtrate was evaporated nearly to dryness, 100 cc. of water were added, and the solution was again evaporated under reduced pressure. The residue was made alkaline with

1.0 N potassium hydroxide and the ammonia distilled off. The residue, after being slightly acidified with acetic acid, was transferred to a small glass evaporating dish and evaporated on a water bath. The amino-acid nitrogen was then determined in the micro apparatus as described by Van Slyke. The apparatus was exactly the same as described by that author,<sup>6</sup> except that the gas burette was smaller, holding only 3 cc.<sup>7</sup> Table II shows the results obtained by this procedure. It will be noted that the results by the vacuum evaporation are very low. A second

TABLE II.

*Comparison of Direct and Vacuum Evaporation of Blood Filtrates Obtained by Trichloroacetic Acid Precipitation. Amino-Acid Nitrogen per 100 Cc. of Blood.*

Material.		Vacuum evaporation.	Direct evaporation.
		mg.	mg.
Ox	blood (defibrinated) .....	1.08	6.20
Sheep	" (oxalated).....	1.95	7.04
Ox	" (defibrinated).....	1.85	6.92
"	" " .....	2.40	6.16
"	" " .....	3.50	6.92
"	" " .....	3.57	6.09
Sheep	" " .....	2.74	6.89
Ox	" + amino-acids.....	4.31	12.59
"	" " .....	3.25	12.71
"	" (defibrinated).....	1.69	6.43

method of removing the trichloroacetic acid was therefore tried. The original filtrate was put into a flask, a drop of alizarin indicator was added and the liquid brought to boiling. It was then kept boiling very slowly for 20 to 45 minutes until the indicator showed that the trichloroacetic acid had been removed. Enough 1.0 N potassium hydroxide was added to make the liquid distinctly alkaline and the ammonia removed by boiling from 1 to 2 minutes. It was then slightly acidified with acetic acid, boiled down further, and quantitatively transferred to a small evaporating dish. After evaporating to a small volume, the

<sup>6</sup> Van Slyke, *J. Biol. Chem.*, 1913-14, xvi, 121.

<sup>7</sup> Van Slyke, *J. Biol. Chem.*, 1915, xxiii, 407.

amino-acid nitrogen was determined. Table II compares the results obtained by the vacuum evaporation and the direct evaporation method.

The low figures by the vacuum distillation procedure required further study. The two processes were therefore reversed; *i.e.*, after evaporating *in vacuo* and before making alkaline, the residue in the distilling flask was transferred to a Florence flask, 2.5 per cent trichloroacetic acid was added to approximately the original volume, and the direct evaporation procedure applied. The residue from the direct evaporation was in turn evaporated *in vacuo* after making to volume with 2.5 per cent

TABLE III.

Vacuum evaporation.	Vacuum evaporation followed by direct evaporation.	Direct evaporation.	Direct evaporation followed by vacuum evaporation.
<i>A. Amino-Acid Nitrogen per 100 Cc. of Blood.*</i>			
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1.79	6.33	7.13	1.91
0.71	6.92	7.08	
3.15	7.22	7.24	
2.17	7.12	6.15	3.60
2.63	7.11	6.17	3.40
<i>B. On Pure Alanine Solution (Calculated 1.2 Mg. of Amino-Acid Nitrogen)</i>			
0.63	1.14	1.16	
0.66	1.18	1.22	

\* Different samples of ox blood were used.

trichloroacetic acid. In the case of vacuum evaporation the results were invariably lower in the same ratio as obtained before. Table III summarizes the results. Each series was run on the same filtrates, obtained by precipitating large amounts of blood with trichloroacetic acid as described above.

Solutions of pure alanine were also tried under the same conditions. To 5 cc. of a solution of pure alanine 200 cc. of 2.5 per cent trichloroacetic acid were added and then treated as before; *i.e.*, (a) vacuum evaporation, (b) direct evaporation, and (c) vacuum evaporation followed by direct evaporation with 200

cc. of 2.5 per cent trichloroacetic acid. Table III B shows these results.

The differences, although not so pronounced as in the blood filtrates, show that the vacuum evaporation gives consistently lower results. This is apparently due to the fact that the trichloroacetic acid is not entirely removed by the vacuum evaporation and enters into some combination with the amino-acids which prevents their quantitative reaction in the Van Slyke method. If pure alanine solutions, after being concentrated to a small volume, are treated with solid trichloroacetic acid and allowed to stand at 40–80°C., they invariably give figures for amino-acid nitrogen which are from 10 to 30 per cent lower than the theoretical. The same observation was made on blood filtrates when treated with solid trichloroacetic acid after free evaporation.

A comparison between the methyl alcohol precipitation method and the trichloroacetic acid procedure was then made. 30 to 50 cc. of blood were allowed to stand with urease for  $\frac{1}{2}$  hour, then precipitated with nine times the volume of alcohol. After standing for 2 hours the precipitate was filtered off by suction, the filtrate treated with a little alcoholic  $\text{ZnCl}_2$  solution, and filtered again. An aliquot portion of the filtrate was evaporated on a water bath to drive off the alcohol, and a little water was added. The liquid was made alkaline with 1.0 N KOH and boiled to drive off the ammonia. It was then acidified with acetic acid, evaporated, and the amino-acid nitrogen determined.

The precipitation with trichloroacetic acid was carried out as described before. Table IV compares the results obtained by the two methods on different bloods. The recovery of amino-acids added to the blood by the two procedures was also investigated. The amino-acid solutions used were obtained by hydrolyzing pure casein with strong HCl as described by Fischer.<sup>3</sup> After removing the HCl as far as possible by vacuum distillation, the solution was diluted to a convenient concentration of amino-acid nitrogen and treated by the Van Slyke method. As ammonia is formed in the hydrolysis of the casein, it is necessary to remove this before the determination of the amino-acid nitrogen is made.

<sup>3</sup> Fischer, E., Untersuchungen über Aminosäuren, Polypeptide und Proteine, Berlin, 1906, 55.

TABLE IV.

*Comparison of Methyl Alcohol and Trichloroacetic Acid as Blood Precipitants for the Determination of Amino-Acids. Amino-Acid Nitrogen per 100 Cc. of Blood.*

Material.			Methyl alcohol.	Trichloroacetic acid.
			mg.	mg.
Ox	blood (defibrinated)	.....	7.79	8.91
"	"	"	7.14	7.09
"	"	"	5.74	6.16
"	"	"	6.54	6.66
Sheep	"	"	6.86	6.89
"	"	"	6.09	8.19
Calf	"	(oxalated)	5.17	6.66
Ox	"	+ amino-acids	10.44	12.59
"	"	(oxalated)	5.13	6.89
"	"	(defibrinated)	5.07	6.43
"	"	+ amino-acids	9.53	12.71
Sheep	"	(oxalated)	3.72	7.82
"	"	"	4.25	7.79

TABLE V.

*Recovery of Amino-Acids Added to the Blood by Methyl Alcohol and by Trichloroacetic Acid Precipitation. Amino-Acid Nitrogen per 100 Cc. of Blood.*

Material.	Methyl alcohol.				Trichloroacetic acid.			
	Blood.	Blood + amino-acid.	Difference.	Calculated.	Blood.	Blood + amino-acid.	Difference.	Calculated.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Ox blood	5.13	10.44	5.31	6.28	6.89	12.59	5.70	6.28
" "	5.07	9.53	4.46	6.28	6.43	12.71	6.24	6.28

The results obtained by the methyl alcohol precipitation method are invariably lower, and the recovery of added amino-acids is not so complete as in the case of the trichloroacetic acid procedure (Greenwald<sup>4</sup>).

The latter procedure gives satisfactory results, but is somewhat troublesome in certain stages of the manipulation. Filtration is very slow, and the alternative process of centrifuging such large



volumes (300 to 500 cc.) is not always convenient. Another method was therefore sought.

The main difficulty with most precipitation methods is that the filtrates contain too large an amount of solids in solution to permit evaporation to the small volume necessary for the final determination. Therefore a procedure was adopted providing for preliminary coagulation of the proteins by heat in a faintly acid solution and evaporation of the filtrates to a small volume. The trace of proteins escaping the first precipitation is then removed by a precipitant which does not appreciably increase the amount of salts in the final solution. The heat coagulation was carried out as suggested by Benedict.<sup>9</sup> The following procedure is recommended.

Into a flask introduce approximately 0.3 gm. of ground soy bean (20 mesh), add 3 to 5 cc. of water and 1 cc. of a 3 per cent solution of  $\text{NaH}_2\text{PO}_4$ , and let stand for a few minutes with occasional shaking. Run in a measured amount of blood (from 30 to 50 cc.) and let stand at room temperature for 30 minutes.

Heat 0.01 N acetic acid to boiling in a casserole, using five volumes of acid for one volume of blood. Run the blood from the flask slowly into the boiling acid and with constant stirring boil for  $\frac{1}{2}$  minute. Add the same amount of boiling water, using this also to rinse the flask. Boil with stirring for 1 minute. Filter through a folded filter and wash the casserole three times with small portions of water (30 cc.), heating the water in the casserole in which the original coagulation took place and using a rubber-tipped stirring rod. The filtrate is boiled down rapidly over a free flame to about 10 cc. in a casserole. The contents of the casserole are now quantitatively transferred to a small graduated flask or cylinder, choosing the size so as to obtain nearly the volume of the original blood. Wash the casserole with the smallest possible amount of hot water three times. The volume in the flask or cylinder, after the final wash water has been added, should not be more than about three-fourths of the final volume.

At this stage of procedure different protein precipitants were tried. The first was a solution of colloidal iron (5 per cent Merck).

<sup>9</sup> Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

This method, while removing the proteins completely, requires a little experience to obtain filtrates which can be evaporated to a very small volume without getting cloudy. It was therefore abandoned for the present. The next precipitant tried was 7 per cent phosphotungstic acid (Merck) in 2 per cent HCl solution, the phosphotungstic acid in turn being removed by gradual addition of small amounts of solid  $\text{Ba}(\text{OH})_2$ .

This method gives very coarse, easily filterable precipitates, but the results are too low. As the phosphotungstic acid precipitant may possibly be used in some later work, some of the results obtained are given in Table VI. The results are compared with another procedure which will be discussed shortly.

TABLE VI.  
*Amino-Acid Nitrogen per 100 Cc. of Blood (Sheep).*

Heat coagulation followed by phosphotungstic acid precipitation.	Heat coagulation followed by trichloroacetic acid precipitation.
<i>mg.</i>	<i>mg.</i>
4.64	7.60
4.13	
4.37	
4.48	
3.25	
3.12	6.34
Blood..... 3.18	
" + amino-acid..... 8.60	
Difference..... 5.42	
Calculated..... 9.32	

The third precipitant tried was trichloroacetic acid followed by kaolin, as suggested by Greenwald. The filtrate from the heat coagulation after being evaporated to a small volume and transferred to a graduated flask or cylinder is treated with trichloroacetic acid. Introduce into the graduated flask enough solid trichloroacetic acid to make an approximately 3 per cent solution. For this purpose the acid is either weighed out on a small scale in a little glass scoop or watch-glass and washed into the cylinder with a little water, or if several determinations are made, a 50 per cent solution of trichloroacetic acid is kept on hand and the corresponding amount of this solution is added with a Mohr

pipette. After making the solution up to volume, let it stand for 20 to 30 minutes. Shake with 2 gm. of kaolin, centrifuge, and run the supernatant liquid through a dry filter paper. A little kaolin always sticks to the side of the centrifuge tube above the liquid level and is carried along when the liquid is poured out. An aliquot part of the filtrate is transferred to a small flask, and a few beads and a drop of alizarin indicator are added. The liquid is brought to boiling over a micro burner and kept boiling very slowly (simmering) until the indicator turns.

The flask is removed from the flame and enough (1 to 2 cc.) of 1.0 N potassium hydroxide is added to make the liquid distinctly alkaline. Boil for 1 to 2 minutes, taking care that it does not boil over, because at this stage slight frothing and bumping occur. Make distinctly acid with acetic acid and boil down to the smallest possible volume. The liquid is now ready for the amino-acid apparatus. It is either transferred directly to the burette of the Van Slyke apparatus, washing the flask with very little water, or first transferred to a small accurately graduated test-tube and made to a definite volume. From this tube duplicates can be measured out by means of the burette of the amino-acid apparatus.<sup>10</sup> The latter procedure is especially recommended where large amounts of blood are available.

Table VII A shows a comparison between the direct trichloroacetic acid precipitation and the heat coagulation followed by the trichloroacetic acid precipitation. The corresponding results were obtained from the same blood each time. Table VII B shows the recovery of amino-acids added to the blood.

<sup>10</sup> The heat-trichloroacetic acid method gives filtrates which rarely exhibit any tendency to froth, when shaken in the deaminizing bulb. Should frothing occur, for some reason, caprylic alcohol, as recommended by Van Slyke, is very efficient. The best caprylic alcohol which we have been able to obtain at present gives such high corrections for the blanks that it should not be used without purification. For that purpose the alcohol is shaken twice (best in a separatory funnel) with a mixture of glacial acetic acid and  $\text{NaNO}_2$  solution (30 gm. in 100 cc. of  $\text{H}_2\text{O}$ ), the acid and the nitrate being in the proportion 1 : 5. The alcohol is then washed with a little water two or three times, transferred to a distilling flask, a very small fraction of  $\text{NaOH}$  added, and distilled under reduced pressure. The caprylic alcohol so purified shows a negligible increase in the blank figures.

TABLE VII.  
A. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Trichloroacetic acid precipitation.	Heat coagulation followed by trichloroacetic acid precipitation.
	mg.	mg.
Sheep blood (oxalated).....	7.82	7.43
Sheep blood (oxalated).....	7.79	7.60
Sheep blood (oxalated).....	7.50	6.34
Calf blood (oxalated).....	7.60	7.24
Pig blood (oxalated).	8.33	8.37

B. Recovery of Added Amino-Acids.

	Blood.	Blood + amino-acid.	Difference.	Calculated.	Blood.	Blood + amino-acid.	Difference.	Calculated.
Sheep blood (oxalated).....	7.50	16.33	8.83	9.32	6.34	15.84	9.50	9.32
Calf blood (oxalated).....	7.60	16.85	9.25	9.40	7.24	16.44	9.24	9.40

The use of heat coagulation prior to amino-acid determination might seem objectionable on account of possible hydrolysis of protein during the process. Greenwald has shown in his publication that no splitting off of nitrogen takes place with his procedure, but here the first precipitation takes place in the cold. A glance at Table VII A shows that the heat coagulation-trichloroacetic acid procedure gives even slightly lower results than the direct Greenwald procedure. Recently Folin and Denis<sup>11</sup> have stated that, "All reagents involving heating are useless, because by heat (half an hour in a water bath) the nitrogen of normal blood filtrates may be increased to twice the real value." No figures are offered in substantiation of this statement. According to the statement of Folin and Denis, we should expect that the heat coagulation procedure would show much higher results in amino-acid nitrogen because the supposed increase in nitrogen would to a large extent be derived from protein hydrolysis.

<sup>11</sup> Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 492.

The above mentioned comparison seemed convincing, but an additional experiment was made to furnish further proof. Blood was precipitated with methyl alcohol according to Folin and Denis. Another sample of the same blood was precipitated according to Greenwald, and a third part of the blood was coagulated by heat and after evaporation treated with trichloroacetic acid and kaolin exactly as described above. On the filtrates, obtained by these three procedures, Kjeldahl determinations were made, using such volumes of filtrates as to represent about 40 cc. of blood, and the determination was repeated with three different samples of blood. Table VIII shows the results obtained.

TABLE VIII.

*Non-Protein Nitrogen per 100 Cc. of Blood (Pig).*

Methyl alcohol precipitation.	Trichloroacetic acid precipitation.	Heat and trichloroacetic acid precipitation.
mg.	mg.	mg.
22.93	23.88	23.60
29.92	32.03	30.80
28.15	30.81	29.22

## SUMMARY.

The use of alcohol as a blood precipitant in the determination of amino-acid nitrogen in blood is undesirable.

A comparative study is made of several procedures to obtain suitable blood filtrates and their subsequent preparation for final analysis by the Van Slyke method.

The Greenwald method of blood precipitation has been found serviceable for amino-acid nitrogen determination. A modification of this procedure is also described.

Coagulation of blood at a boiling temperature in a weakly acid solution does not increase the filtrate nitrogen, provided the traces of protein which escape coagulation are properly removed.

A study of bloods of different species of animals and of human blood, both normal and pathological, is being made and will be published shortly.

In conclusion I wish to thank Mr. Isaac Neuwirth for his assistance in part of the experimental work.

# STUDIES IN THE COMPARATIVE BIOCHEMISTRY OF PURINE METABOLISM.

## III. THE PRESENCE OF ALLANTOIN IN MAMMALIAN BLOOD.

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(Received for publication, November 3, 1916.)

Since the urine of the great majority of mammalian species contains, as the end-product of purine metabolism, considerable quantities of allantoin, the presence of that substance in the blood also might reasonably be taken for granted. It seemed none the less desirable to convert this inference into certainty by the actual isolation of the substance. The attempt to effect this met with greater difficulty than I anticipated, but was finally so far successful that from the blood of the ox and the pig allantoin was obtained in quantity sufficient for positive identification, while its presence in that of the horse and the sheep was rendered practically certain. Human blood, on the other hand, yielded no evidence of containing even a trace of allantoin.

The method employed for the final isolation of the substance was that of Wiechowski.<sup>1</sup> But before it could be applied to blood it was necessary first to remove the proteins, and then to adopt some means of concentrating the other nitrogenous constituents (or at least the allantoin itself) into a solution of convenient bulk. In preliminary experiments with pig blood I sought to attain these objects by heat coagulation of the proteins, and evaporation of the faintly acid filtrate upon the water bath. The end-product obtained by the application of Wiechowski's method to this concentrate undoubtedly contained allantoin; but the amount was so small, and it was so contaminated by other materials, that its certain identification was hardly possible. Better results followed the adoption

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<sup>1</sup> Wiechowski in Neubauer-Huppert, *Analyse des Harns*, Wiesbaden, 11th edition, 1913, ii, 1076.

with water. The filtrate was acidified with acetic acid, and freed from chlorides by silver acetate. Upon removal of lead, silver, and hydrogen sulfide in the usual way, the final filtrate was concentrated to about 60 cc. and, after careful neutralization with chloride-free sodium hydroxide, was treated with an equal volume of Wiechowski's reagent. The immediate result varied from a flocculent precipitate in the case of ox blood to a faint opalescence in that of the human fluid. In every case a precipitate had settled out by the following morning. When this precipitate was subjected to the usual technique for the isolation of allantoin, it yielded a product highly impure, which it was difficult or impossible to bring to crystallization. It was therefore taken up in a very little water, treated again with a few drops of basic lead acetate, filtered, freed from excess lead, and precipitated a second time with Wiechowski's reagent. The solution resulting from the decomposition of this second precipitate was evaporated in a tiny glass basin, the dry residue moistened with a little water, and set aside to crystallize. When crystals formed they were washed with cold 50 per cent alcohol, which removed some still adherent greasy impurities, dried at 100°, and weighed.

The results obtained can be reported in a very few words. The blood of the *ox* yielded 4.7 mg. of clean well shaped crystals of considerable size and typical form, which melted, simultaneously with a specimen of pure allantoin heated alongside, at 232°C. The *pig* blood gave 3.4 mg. of crystalline material; the crystals were smaller, and assumed the shape not of perfectly pure allantoin, but of that substance as obtained by direct crystallization from urine; they showed, nevertheless, a melting point identical with that of the pure material. From the blood of the *horse* there were obtained crystals similar to those of the pig, although visibly inferior in quantity; unfortunately the dish containing them was accidentally broken before they could be weighed or their melting point determined. The *sheep* blood yielded at first only a smear, and it was only after much manipulation that there appeared two microscopic clumps of crystals; they had all the appearance of impure allantoin, but the quantity was too small to be accurately weighed, or even to serve for a satisfactory melting point determination. *Human* blood<sup>5</sup> furnished a minute

<sup>5</sup> The blood examined was a mixed specimen obtained by venesection from two nephritics. It is perhaps worth pointing out, in connection with recent observations by H. G. Wells (*J. Biol. Chem.*, 1916, xxvi, 319), how readily it yielded uric acid. The failure to obtain allantoin from such blood is even stronger evidence of its absence than if the blood had come from normal individuals.

amount of material, which could not be brought by any treatment whatever to yield crystals even remotely resembling allantoin.

The total yield of allantoin from all the specimens utilized was so small that no analysis of the material could be carried out. Nevertheless the identification, in the cases of the ox and the pig, resting as it does upon method of isolation, crystalline form, and melting point, may be regarded as beyond question. With the horse and the sheep, in the absence of a melting point determination, it is not so certain; although to one familiar with the microscopic shapes assumed by allantoin, little doubt upon the point would exist. The complete failure to find allantoin in human blood was of course to be expected.

The maximum yield of 4.7 mg from about 600 cc. of ox blood represents something like 0.8 mg. per 100 cc. This is somewhat higher than the uric acid, and lower than the creatinine content of the same blood. It is of course merely a minimal value. It would indeed be ridiculous, with such a method as has been described, where so many bulky precipitates have an opportunity of adsorbing the substance sought, to lay any stress on quantitative considerations. It may, for instance, be entirely an accident that allantoin was isolated more readily and in greater quantity from the blood of the ox and the pig, than from that of the horse and the sheep. This being admitted, it is nevertheless suggestive that if we arrange the species in descending order in relation to the allantoin obtained, we get a series—ox, pig, horse, sheep, man—which almost coincides with the order taken by the same animals in regard to their capacity to form allantoin from uric acid, as judged by the relative proportions of these substances in the urine. Thus, the uricolytic index of the cow is 93, and of the pig 98, while that of the horse is 88, and that of the sheep only 80;<sup>6</sup> man, of course, has practically no allantoin-forming capacity at all.

Further investigation along the line suggested by these considerations must await the elaboration, if that should prove possible, of a more convenient method for the determination of al-

<sup>6</sup> Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1914, xviii, 403.



lantoin in blood. The only one at present available is so tedious and time-consuming that a repetition, with perhaps larger quantities of blood and a wider range of species, would hardly repay the labor involved. The main object of the present investigation was attained when allantoin, in however small a quantity, was actually isolated from mammalian blood.

## THE IODINE CONTENT OF FOOD MATERIALS.\*

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(Received for publication, November 8, 1916.)

The relation of iodine to thyroid metabolism has received a large amount of study, but the supply of iodine in food materials had been given no systematic attention until taken up by Forbes.<sup>1</sup> What influence its total absence in the diet might have on the development of an animal is problematic, but there has not been a lack of opinion that such an absence or low supply in the foods from certain regions was related to the development of goiter. To this opinion the work of Forbes and Beegle gives no credence. Further, we have learned that the production of hairless and weak pigs in certain regions in the northwestern part of the United States is attributed by some to a low supply or absence of iodine in the ration of brood sows.

Before the work of Forbes and Beegle appeared, we had under way a quantitative survey of the iodine content of a number of foodstuffs, waters, and samples of common salt used by farm stock. The results secured are in agreement with their data and show a low supply of iodine in plant materials and common salts, obtained from widely separate regions. Our only excuse for publishing these results is that they are confirmatory of the work of others, and in addition summarize our experience with certain methods proposed for the quantitative determination of iodine in organic materials.

The first method which we attempted to use was that of Krauss,<sup>2</sup> in which the iodine is determined colorimetrically as  $PdI_2$ . This

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> Forbes, E. B., and Beegle, F. M., *Ohio Agric. Exp. Station, Bull.* 299, 1916.

<sup>2</sup> Krauss, R. B., *J. Biol. Chem.*, 1915, xxii, 115.

method is claimed to detect and determine as low as 0.0001 mg. of iodine in a 2 gm. sample. Though the directions were followed with the greatest care, no iodine, when added as potassium iodide in quantities of 0.0001 to 0.001 mg., could be recovered in the controls, and the method was finally abandoned.<sup>3</sup>

We next went back to the method proposed by Hunter<sup>4</sup> in which the iodine, after fusion and solution, is oxidized to iodate by sodium hypochlorite, the excess of chlorine removed by boiling, the iodine liberated from the iodate by potassium iodide in excess, and titrated with sodium thiosulfate. The results with this method were variable, at times being good and at other times disappointing.

Finally we tried the Kendall<sup>5</sup> method which is as follows:

The substance, finely ground, is evaporated to a syrup in a nickle crucible with 5 cc. of 30 per cent sodium hydroxide and 10 to 15 gm. of solid sodium hydroxide broken into small pieces. The mass is then fused in a muffle furnace and any unoxidized organic matter is removed by small additions of solid potassium nitrate. After cooling, the melt is dissolved in water and transferred to a 500 cc. Erlenmeyer flask. 1 cc. of 10 per cent sodium bisulfite and a few drops of methyl orange are added, and the whole is made just acid with 85 per cent phosphoric acid. A few drops of bromine are added, the flask is well shaken, and the bromine boiled off by 10 minutes of vigorous boiling. About 10 drops of a 5 per cent solution of sodium salicylate are now added, and the flask and its contents cooled in running water. 5 cc. of 10 per cent potassium iodide are added, and a little phosphoric acid, if necessary, to bring about the iodide-iodate reaction. The liberated iodine is titrated against standard sodium thiosulfate, using starch paste as indicator. We found arrowroot starch as recommended by Hunter<sup>4</sup> suitable for this purpose. The iodine found must be divided by 6 to give the "original" iodine.

After some experimentation excellent results were obtained. The methyl orange end-point in the acidification with phosphoric acid was elusive, however, and a trial with Congo red showed the latter to be superior. In this case the acid is added until the indicator goes to a distinct blue, not stopping at the first break in the red which is still on the alkaline side. With the

<sup>3</sup> Mr. F. M. Beegle of the Ohio Experiment Station informed me that he also had been unable to obtain good results with the Krauss method.

<sup>4</sup> Hunter, A., *J. Biol. Chem.*, 1909-10, vii, 321.

<sup>5</sup> Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

above method, controls in which 0.1 to 1 mg. of iodine were added, the iodine was always recovered quantitatively. In the actual determination 2 gm. samples of the air-dried feedingstuff were used, and two and sometimes more determinations were made in nearly all cases. The minimum iodine content which Kendall claims can be detected is about 0.005 mg. of "original" iodine in a 2 gm. sample, or even 0.003 mg. of iodine. This much iodine, multiplied by 6 in the determination, gives a perceptible blue color with starch. In titrating these extremely small amounts the thiosulfate, which was standardized daily against potassium acid iodate, was used in various strengths of 0.002 N to 0.0005 N. Nothing is gained by using a strength weaker than 0.001 N, however, as the end-point becomes quite indistinct.

#### SUMMARY OF RESULTS.

Corn meal, tankage, commercial meat scraps, clover hay, alfalfa, cabbage, ground oats, oat meal, oats at the period of flowering, very young oats, June grass, timothy hay, wheat flour, sugar beet, milk powder, oil meal, distillers' grains, wheat gluten, oat straw, wheat straw, rape, cottonseed flour, peas, pea vine, and alfalfa grown in Kansas gave results which, on the whole, exclude the presence of as much as 0.003 mg. of iodine in 2 gm. of the substance. Wheat germ, barley, sweet clover, and Kansas-grown prairie hay possibly showed a trace of iodine, not more, certainly, than 0.005 mg. in a 2 gm. sample. Corn gluten, potato, lettuce, and the two natural waters examined showed a distinct trace of iodine, ranging from 0.003 to 0.01 mg. per 2 gm. of sample or 1 liter of water, respectively. The waters examined were from a local driven well and from Lake Mendota, a small inland lake which supplies this laboratory. 1 liter of water was evaporated to dryness with a little sodium hydroxide and the residue treated in the same manner as were the feeds. The blue color kept coming back at the final titration, however, and the iodine may have been liberated from the added potassium iodide by something other than iodate, as a ferric salt. This determination was originally intended for use with thyroids, which contain little inorganic material, and it is possible that some of the inorganic salts, which are present in rather large

amounts in these waters, rather than original iodine, liberated iodine from potassium iodide.

It was reported that some of the soils and waters in Kansas contain traces of iodine and it was considered of interest to determine the iodine content of some Kansas-grown feeds. Through the courtesy of Professor P. J. Newman of the Kansas State Agricultural College, samples of alfalfa, wheat, oat straw, and prairie hay were obtained and examined with results as noted above.

Samples of rock salt such as are commonly fed live stock were obtained and examined for iodine as follows: 10 gm. samples were dissolved in water and 10 cc. of saturated sodium carbonate solution were added, and 1 cc. of 10 per cent sodium bisulfite. A drop of Congo red brought the samples to the point in the Kendall determination where the phosphoric acid addition is made, and the determinations were finished as above. Controls showed this modification to be suitable for the determination of iodine in common salt. Samples were examined from two mines in Michigan, from New York, Louisiana, Kansas, Ohio, the Pacific Coast, and Salt Lake City. In no case was there any indication of the slightest trace of iodine present.

Attempts were also made to extract the iodine from 25 gm. of corn meal with a mixture of alcohol and acetone in a continuous fat-extraction apparatus, and determine the iodine in the evaporated residue. No iodine was found, however, and in a control only about three-fifths of the added potassium iodide was recovered.

An attempt was made to determine the iodine in 50 gm. of corn meal by adding the sample gradually to a large melt of sodium hydroxide, but the early production of sodium carbonate and subsequent solidification of the residue prevented the determination from being at all satisfactory, and a blank was obtained. A hot water extraction of 100 gm. of corn meal also gave a blank determination.

Distillations of the iodine from large samples suspended in 25 to 50 per cent sulfuric acid solutions were tried, but owing to the excessive frothing they have not yet been satisfactory.

TABLE OF RESULTS.

Substance.	No. of determinations.				Probable presence or absence of iodine.
	Total.	No iodine.	Trace.	0.003 to 0.01 mg. of iodine.	
Meat scraps.....	3	2	1		No iodine.
Tankage.....	2	1	1		" "
Corn meal.....	2	1	1		" "
Corn stover.....	2	2			" "
Cabbage.....	2	1	1		" "
Clover hay.....	2	2			" "
Very young oats.....	1	1			" "
Oats in flowering.....	1	1			" "
Ground oats.....	2	2			" "
Oat meal.....	2	2			" "
June grass.....	2	2			" "
Alfalfa.....	2	2			" "
Timothy.....	2	2			" "
Wheat flour.....	1	1			" "
Sugar beet.....	2	2			" "
Milk powder.....	2	2			" "
Wheat germ.....	1		1		Possibly a trace.
Distillers' grains.....	1	1			No iodine.
Wheat gluten.....	1	1			" "
Corn gluten.....	6	1	1	4	Undoubtedly a trace.
Barley.....	4	2	1	1	Possibly a trace.
Barley straw.....	3	2		1	No iodine.
Potato.....	3		1	2	Probably a trace.
Oat straw.....	1	1			No iodine.
Wheat straw.....	1	1			" "
Oil meal.....	1	1			" "
Lettuce.....	3		2	1	Probably a trace.
Rape.....	1	1			No iodine.
Cottonseed flour.....	1	1			" "
Peas.....	2	2			" "
Pea vine.....	2	2			" "
Sweet clover.....	1		1		Possibly a trace.
Water samples.					
Well water.....	2	1		1	Probably " " (?)
Lake Mendota water...	3	1		2	" " " (?)
Kansas samples.					
Prairie hay.....	1		1		Possibly " "
Wheat.....	1			1	Probably " "
Oat straw.....	1			1	" " "
Alfalfa.....	1	1			No iodine.

TABLE OF RESULTS—*Concluded.*

Substance.	No. of determinations.				Probable presence or absence of iodine.
	Total.	No iodine.	Trace.	0.003 to 0.01 mg. of iodine.	
Salt samples.					
Michigan, No. 1.....	2	2			No iodine.
"    "    2.....	2	2			"    "
Louisiana.....	2	2			"    "
New York.....	2	2			"    "
Kansas.....	2	2			"    "
Ohio.....	2	2			"    "
California .....	2	2			"    "
Utah.....	2	2			"    "

## DISCUSSION.

The above results are not absolute, as the table shows, but they are the best interpretation of the data obtained. That is to say, the results were not always in agreement—a blank and a trace of iodine being sometimes found in the same sample. The table gives the detailed results of the determinations. To test the effect of maturity, the oat plant was examined at three stages of growth, but negative results were obtained in every case.

It might be supposed that the objection to the method as applied to water would also apply to some of the feeds, but repeated controls showed that the method was quite applicable. Since 2 gm. samples were used, and 0.003 mg. of original iodine gives a blue color to the final solution, the samples which gave a blank had considerably less than one part of iodine in 650,000. In the case of the salts where 10 gm. samples were used, the amount of iodine was less than one part in 3,250,000. For the waters the amount was about one-hundredth of this. The substances, which showed a trace of iodine, had from about one part in 650,000 to one part in 200,000. Nearer than this it is impossible to say, the results, perhaps, being more qualitative than quantitative. There is, however, substantial agreement between these results and those secured by Forbes and Beegle.

## SUMMARY.

1. Three methods for the determination of iodine in organic matter have been compared, with the result that the method proposed by Kendall was found to be by far the most accurate.

2. Iodine determinations have been made on many feeding materials, two natural waters, and several rock salts. In the majority of samples no iodine could be detected; some showed a trace of iodine to be present.

3. It would appear that the presence of iodine in feeding materials of vegetable origin is accidental and serves no necessary nutritive function in the plant. Further, the iodine requirements of animals must of necessity be met by the traces that occur in plant materials, waters, etc.

I wish to express my gratitude to Professor E. B. Hart of this Station, for the suggestion of this research and for much helpful advice and criticism.





# COLORIMETRIC METHODS FOR DETERMINING SERUM CHOLESTEROL.

By PAUL G. WESTON.

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(Received for publication, November 4, 1916.)

Colorimetric methods have been used by a number of workers for the quantitative determination of cholesterol in human blood. Weston (1) and Schmidt (2) used the Salkowski reaction. Grigaut (3), Autenrieth and Funk (4), Henes (5), Csonka (6), Bloor (7), Gettler and Baker (8), and others used the Liebermann-Burchard reaction. Each author used a different method of extraction. Autenrieth and Funk made fourteen determinations by their method and found an average of 1.278 mg. of cholesterol per 1 cc. of serum. Henes (5), testing some of the same serum by the Weston-Kent (9) method, found an average of 1.267 mg. per 1 cc. Weston (10) added known quantities of cholesterol to serum and recovered it quantitatively by the Weston-Kent method. Henes in the University of Freiburg used the Weston-Kent method for extraction and the Grigaut method for color determination; Windaus used his digitonin method, and Autenrieth used the Autenrieth-Funk method. The results were almost identical.

The experiment here described was undertaken in order to have a basis of comparison for the different methods both of extraction and of color determination. Starting with 100 cc. of human serum, I divided the quantity into two equal parts and added to one part 50 mg. of pure cholesterol dissolved in ether. The flask containing the serum-ether mixture was gently shaken from time to time until the ether had evaporated. By this method an even distribution of the cholesterol in the serum was obtained. A portion of each of these sera was extracted by each of six methods and each of the twelve resulting extracts

was tested by three methods. The following extraction methods were employed.

1. *Bloor Method*.—Three volumes of serum were added to seventy-five volumes of a mixture of alcohol and ether (alcohol, three parts; ether, one part) in a flask, and the mixture was heated to boiling. It was allowed to cool, after which sufficient alcohol-ether mixture was added to bring the volume to 100 and the extract was filtered. At this point the filtrate was divided into two parts. The first part was evaporated to dryness by means of gentle heat strictly according to Bloor's instructions, and the residue was taken up in chloroform for testing. The second was boiled with potassium hydrate for 2 hours and treated subsequently as in the Weston-Kent method.

2. *Autenrieth-Funk Method*.—Serum was digested on a water bath for 2 hours in ten volumes of 25 per cent potassium hydrate solution. The resulting liquid was extracted by adding to it twice its volume of chloroform and shaking vigorously for 5 minutes. The chloroform was then drawn off and a fresh lot added. This was repeated four times. The extract was dehydrated with anhydrous sodium sulfate and the clear solution used for testing.

3. *Weston-Kent Method*.—Serum was extracted with ten volumes of 95 per cent alcohol and kept at a temperature of 60°C. for 24 hours. The alcohol was filtered off and the residue washed twice with boiling alcohol. The residue was then further extracted for 24 hours with ether. The ether and alcohol extracts were combined and the mixture boiled with a few grams of potassium hydrate for 2 hours, at the end of which time the fluid had evaporated to a very small volume. A saturated solution of calcium hydrate was added and the precipitate which formed was collected on a filter, washed with calcium hydrate solution, dried, and extracted with chloroform.

4. *Csonka Method*.—2 cc. of serum, 20 cc. of alcohol, 4 gm. of potassium hydrate, and 2 cc. of a 10 per cent solution of barium chloride were placed in a flask of special construction and boiled under a reflux condenser for 1 hour. The mixture was extracted with ether and the extract washed with water until the wash water was no longer alkaline. The ether extract was then filtered into a flask, the ether was distilled off, and the residue taken up in chloroform for testing.

5. *Gettler and Baker Method*.—5 cc. of serum were allowed to run onto a loose coil of fat-free absorbent paper; this was dried over  $P_2O_5$  at 55–60°C. in *vacuo* for 16 hours (over night). The coil was extracted in a Soxhlet with anhydrous ether for 5 hours. The ether was evaporated and the residue taken up in chloroform for testing.

6. *Author's Method*.—2 cc. of serum and 20 cc. of 25 per cent potassium hydrate solution were boiled on a hot plate for 2 hours. Water was added occasionally. At the end of 2 hours the solution was evaporated almost to dryness and a saturated solution of calcium hydrate added. The resulting precipitate was collected on a filter, dried, and extracted with chloroform.

*Methods of Testing the Extracts.*

1. *Bloor Method.*—A portion of each extract representing 0.3 cc. of serum was brought to 5 cc. by the addition of chloroform. 2 cc. of acetic anhydride and 0.1 cc. of sulfuric acid were added and the mixture was shaken. A standard tube was made by dissolving 0.0027 gm. of cholesterol in 10 cc. of chloroform. 4 cc. of acetic anhydride and 0.2 cc. of sulfuric acid were added and the mixture was shaken. The tubes were left in the dark at room temperature for 15 minutes. Readings were made in the Autenrieth-Königsberger colorimeter.

2. *Autenrieth-Funk Method.*—A second portion of the extracts, representing 0.1 cc. of serum, was treated in the same manner as above described except that immediately after shaking the mixture of chloroform, acetic anhydride, and sulfuric acid, the tubes were placed in a water bath at 35°C. for 15 minutes. The standard tube contained 0.0012 gm. of cholesterol. Comparisons were made in the Autenrieth-Königsberger colorimeter.

3. *Weston Method.*—A third portion of the extracts was placed in small tubes 100 x 10 mm. and of exactly the same diameter. Three dilutions of each extract were made representing 0.075, 0.1, and 0.125 cc. of serum. The contents of each tube were brought to 1 cc. by the addition of chloroform. In another set of similar tubes were placed quantities of cholesterol varying from 0.0001 to 0.0003 gm. in 1 cc. of chloroform, increasing by 0.000025 gm. 0.1 cc. of sulfuric acid was added to each tube and the tube was thoroughly shaken. All tubes were placed in the dark for 30 minutes, after which 1 cc. of chloroform was added to each tube. They were again placed in the dark for 15 minutes, after which comparisons were made and the amount of cholesterol determined.

A duplicate set of this extract was set up and treated in the same manner except that the standard was made up in 6 cc. of chloroform. Comparisons were made in the Autenrieth-Königsberger colorimeter. The findings by the colorimeter were the same as the results obtained by the use of the small tubes.

The results of all the tests are shown in Table I.

It may be seen that the color values obtained by Bloor's method are remarkably inconsistent and that the addition of cholesterol to the serum is in no case indicated by the readings. Whatever the method of extraction, there is nothing to show that the added cholesterol was recovered. The Autenrieth-Funk method shows clearly the recovery of the added cholesterol in every instance except in the extracts obtained by Bloor's method.

The values obtained by the author's colorimetric method were practically identical with those obtained with the Autenrieth-Funk method.

TABLE I.  
Cholesterol per 1 Cc. of Serum.

Method.	Serum.	Colorimetric methods.		
		Bloor.	Autenrieth-Funk.	Weston.
		mg.	mg.	mg.
Bloor.....	A*	2.12	2.40	
	A**	1.89	1.62	1.73
" .....	B	1.71	2.88	
	B**	1.56	1.88	2.70
Autenrieth-Funk.....	A	2.12	1.86	1.84
	B	1.89	2.87	2.85
Weston-Kent.....	A	3.83	1.84	1.85
	B	3.11	2.86	2.85
Csonka.....	A	2.16	1.85	1.84
	B	2.22	2.87	2.83
Gettler-Baker.....	A	3.51	1.83	1.84
	B	3.00	2.85	2.85
Author.....	A	3.83	1.85	1.85
	B	3.96	2.87	2.86

\* Serum A is plain serum; Serum B is the same with 1 mg. of cholesterol added to each cc.

\*\* Extract saponified by Weston-Kent method.

It will be noted that Bloor's method of extraction did not give consistent results even when tested by other methods, and that the saponification of the extract did not affect the results appreciably. In the writer's hands Bloor's method was useless.

Inasmuch as the other five methods of extraction give identical results, the selection of a method is a matter of personal preference. I prefer the sixth on the ground that it is simple and economical.

Bloor and Autenrieth and Funk mention difficulties arising from the admixture of a yellowish or brownish tint to the green solution in the chloroform containing the serum extract. The latter authors by dehydrating the chloroform with anhydrous

sodium sulfate were nearly always able to remove the interfering color. Csonka overcame the difficulty by adding barium chloride to the mixture before saponification. Bloor states that, "If the evaporation of the untreated alcohol-ether extract was carried out carefully so as to avoid heating the residue after it had reached dryness, the chloroform extract was colorless and after treatment gave excellent readings." Mueller (11) used Bloor's method and was unable to obtain a green color free from brown even when the extract was evaporated before an electric fan. I have had a similar experience, and in eighty extractions was not once able to obtain a specimen wholly free from a brownish color.

#### SUMMARY.

A quantity of human serum was divided and cholesterol was added to one part in the proportion of 1 mg. to 1 cc. Portions of both the pure serum and the treated serum were extracted by different methods and the extracts were tested by different methods for cholesterol. The author failed to obtain valid results by Bloor's method. The extraction methods of Autenrieth and Funk, Weston and Kent, Csonka, Gettler and Baker, and Weston showed uniformly consistent results, the added cholesterol being recovered quantitatively in each case. The Autenrieth-Funk and the author's colorimetric methods gave equally satisfactory results. A new method of extraction is described.

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# THE MODE OF ACTION OF UREASE AND OF ENZYMES IN GENERAL.

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(Received for publication, November 18, 1916.)

Van Slyke and Cullen, in a paper with the above title, deduced a general expression for the kinetics of enzyme action.<sup>1</sup> In a discussion of this question between Professor Pegram, of Columbia University, and the writer, it appeared that in the development of the equations an assumption was introduced which unfortunately limits their validity and applicability to definite conditions which are realized only in special cases.

Van Slyke and Cullen follow the time required for an enzyme molecule to complete the cycle of the reaction; in the example used, the sum of the times required to combine with a urea molecule, and to throw it off as ammonia and carbon dioxide. This length of time may be perfectly definite for a molecule of enzyme going through the cycle once, but with a large number of enzyme molecules it is necessary to consider the *average* time of an enzyme molecule in passing through the cycle.

If, in considering such a large number, the same fraction of the number of molecules is considered to be in each state of the reaction, that is, a part is going through the first stage of combination with the urea molecules, and a part through the second stage of decomposition of the enzyme-urea complex into enzyme, ammonia, and carbon dioxide, then the deductions of Van Slyke and Cullen hold. As the reaction proceeds, with definite initial quantities of enzyme and substrate, the absolute quantity of enzyme remains constant but the quantity of substrate decreases. Under these conditions it is evident, from a consideration of the kinetics of the reaction, as the quantity of substrate

<sup>1</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 146.



decreases, with the same absolute amount of enzyme in the sphere of action, that a decreasing fraction of the number of enzyme molecules will be passing through the first stage of the reaction in a definite time interval, or that the average time of the first stage of the reaction for an enzyme molecule will be altered.

The second step of the reaction, decomposition of the complex, follows the monomolecular reaction velocity law in which the amount decomposed or reacting is proportional to the amount present. The average time each enzyme molecule remains in this combination is constant. The actual concentration of this intermediate compound is, however, changing. The total average time an enzyme molecule occupies in passing through a complete cycle of the reaction is made up, therefore, of the average time required to combine with a substrate molecule, which may be variable as the concentration of the substrate changes, plus the average time of decomposition of the complex, which is constant.

If a steady state exists in the reaction the deductions of Van Slyke and Cullen will conform to the facts. This steady state can occur only if a constant fraction of the total number of enzyme molecules is passing through the first stage of the reaction. This is true in the definite case of a large excess of substrate present in the reaction mixture, but as far as can be foretold, in no other simple case.

The fact that the calculated values, using Van Slyke and Cullen's equation, agree with the experimental results cannot be used as proof for the validity of the assumptions involved in the theoretical deductions. The constants of the equation are obtained from the experiments to which the equation is later applied.

# THE MODE OF ACTION OF UREASE AND OF ENZYMES IN GENERAL.

BY DONALD D. VAN SLYKE AND GLENN E. CULLEN.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, November 18, 1916.)

Our equation, contrary to the statement of Falk in the preceding paper, is not, as it appears to us, limited by the assumption that "a constant fraction of the total number of enzymes is passing through the first stage of the reaction." The equation expresses the following conditions: that the enzyme performs the two acts, of combination with and decomposition of the substrate, in sequence, not simultaneously, and that the period required for combination varies directly as the dilution of the substrate (urea). As corollaries it also expresses the following: first, since the two acts occur in sequence, the proportion of enzyme molecules engaged in each varies directly as the relative periods required for the respective reactions; second, since the average period of combination increases with substrate dilutions, the *proportion of enzyme molecules in the first stage (i.e., engaged in combining with or waiting to combine with substrate) increases continuously with increasing substrate dilution*. The equation as it stands, therefore, not only covers the condition of a variation in the relative proportions of enzyme in the two states of activity, but specifically expresses the manner in which the proportions vary under the influence of changing substrate concentration.

It is a pleasure to acknowledge Dr. Falk's courtesy in discussing with us his criticism prior to its publication, so that his view and ours on the point where we failed to agree can be here presented together.



# THE INFLUENCE OF THE COAGULATION BY RENNIN UPON THE GASTRIC DIGESTION OF MILK PROTEINS.

BY J. T. LEARY AND S. H. SHEIB.

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(Received for publication, November 9, 1916.)

Owing to the divergent results obtained by previous workers regarding the influence of the coagulation by rennin upon the subsequent gastric digestion of milk proteins, this study was undertaken with the view of ascertaining if possible wherein the great differences exist. According to Hawk,<sup>1</sup> coagulation of the casein by rennin greatly retarded subsequent action of pepsin. In his experiments no foreign substance was added to the digestive mixtures. Abderhalden and Kramm,<sup>2</sup> using a different method wherein sodium oxalate was added to prevent the coagulation, found that thereby the activity of the pepsin was lessened, but to a very slight degree. This result they explained by the adsorption of the pepsin by the paracasein with a resulting digestion from both within and without the coagulum.

## PROCEDURE.

In the following experiments Lilly's scale pepsin was employed, in an acidity of 0.2 per cent hydrochloric acid. To prevent coagulation 2 per cent sodium oxalate was used and toluene was added as a preservative. The digestions were incubated at 38° for varying periods of time. Whole milk was used in all the experiments.

At the conclusion of each period of digestion, the already acid solution was saturated with sodium chloride in substance, heated to boiling, and filtered hot. The coagulum was washed with

<sup>1</sup> Hawk, P. B., *Am. J. Physiol.*, 1903-04, x, 37.

<sup>2</sup> Abderhalden, E., and Kramm, F., *Z. physiol. Chem.*, 1912, lxxvii, 462.

hot 10 per cent sodium chloride solution to free it from adhering non-coagulable matter and the filtrate made up to 200 cc.

When milk itself is so treated a water-clear filtrate is obtained which gives no precipitate with either nitric acid or Roberts' reagent. Milk and boiled pepsin similarly treated give the same results.

In the experiments with boiled milk the heating was conducted under a reflux with constant agitation, which procedure prevents the formation of a pellicle, particularly if the agitation is continued until the milk cools to room temperature. The nitrogen content of the coagulum and of the filtrate was determined by the Kjeldahl method. The Sørensen titration method was employed to determine the amino nitrogen content of the filtrate.

*Series I.*

1.	cc.	2.	cc.
Milk.....	10	Milk.....	10
H <sub>2</sub> O.....	10	Sodium oxalate.....	10
Pepsin solution.....	5	Pepsin solution.....	5
1.2 per cent HCl.....	5	1.2 per cent HCl.....	5

Pepsin solution, 250 mg. in 100 cc.

1. Permitted to clot before adding acid.

2. Clotting prevented by addition of oxalate solution.

The periods of digestion were as follows:

Nitrogen.

$\frac{1}{2}$  hr. digestion.

	1. mg.	2. mg.
Residue.....	23.24	42.98
Filtrate.....	34.58	14.56
Amino N.....	1.568	0.448

1 hr. digestion.

Residue.....	21.7	43.4
Filtrate.....	35.84	14.56
Amino N.....	1.624	0.56

$1\frac{1}{2}$  hr. digestion.

Residue.....	18.62	41.44
Filtrate.....	39.2	16.8
Amino N.....	2.016	0.56

2 hr. digestion.

Residue.....	16.9	39.62
Filtrate.....	40.83	18.76
Amino N.....	2.086	0.533

The results obtained in this series clearly show the relatively poorer peptolytic action upon milk in which sodium oxalate has been used to prevent coagulation.

In Series II boiled and unboiled milk were digested in the same manner.

<i>Series II.</i>							
1.	Raw.	2.	3.	Boiled.	4.		
	cc.		cc.		cc.		cc.
Milk.....	10	Milk.....	10	Milk.....	10	Milk.....	10
H <sub>2</sub> O.....	10	Oxalate.....	10	H <sub>2</sub> O.....	10	Oxalate.....	10
Pepsin.....	5	Pepsin.....	5	Pepsin.....	5	Pepsin.....	5
1.2 per cent		1.2 per cent		1.2 per cent		1.2 per cent	
HCl.....	5	HCl.....	5	HCl.....	5	HCl.....	5

Pepsin solution, 1 gm. in 100 cc.

1 and 3. Clot was permitted to form before adding acid.

2 and 4. Clotting prevented by oxalate.

The periods of digestion were as follows:

#### Nitrogen.

##### 1 hr. digestion.

	1. mg.	2. mg.	3. mg.	4. mg.
Residue.....	15.12	43.24	14.14	41.72
Filtrate.....	44.80	17.92	47.04	19.04
Amino N.....	2.80	1.12	2.66	1.26

##### 2 hr. digestion.

Residue.....	14.42	33.74	19.46	26.18
Filtrate.....	45.92	26.88	41.44	32.48
Amino N.....	3.36	1.40	3.36	2.24

##### 3 hr. digestion.

Residue.....				
Filtrate.....	42.56	42.00	48.16	45.92
Amino N.....	2.80	2.24	3.08	2.80

##### 4 hr. digestion.

Residue.....	15.40	25.20	15.00	28.64
Filtrate.....	49.28	37.24	51.52	45.92
Amino N.....	3.64	2.24	3.36	1.96

In this series the retarded digestion in the oxalate solutions is pronounced. In Experiment 3 this retardation is least evident. The digestion of boiled milk was slightly more rapid than that of raw milk.

A different procedure was followed in Series III. No foreign substance, *i.e.*, no sodium oxalate, was added to the digestive mixtures. The formation of a curd of paracasein was prevented by a change in the manner in which the various ingredients were added.

## Series III.

	1. cc.		2. cc.		3. cc.
Milk.....	10	Milk.....	10	Milk.....	10
H <sub>2</sub> O.....	10	H <sub>2</sub> O.....	10	H <sub>2</sub> O.....	10
Pepsin.....	5	1.2 per cent HCl...	5	A mixture of equal parts of 1.2 per cent HCl and pepsin solution.....	
1.2 per cent HCl..	5	Pepsin.....	5		

Pepsin solution, 250 mg. in 100 cc.

1. Pepsin permitted to coagulate milk before addition of acid.

2. Acid added to precipitate casein before addition of pepsin.

1. Coagulation took place in about 2 minutes, forming a thick heavy curd.

2 and 3. No clot of paracasein was formed, the mixtures remaining practically homogeneous.

## Nitrogen.

 $\frac{1}{2}$  hr. digestion.

	1. mg.	2. mg.	3. mg.
Residue.....	44.80	25.20	25.90
Filtrate.....	13.44	35.84	33.60
Amino N.....	0.336	1.29	1.40

## 1 hr. digestion.

Residue.....	40.74	19.32	20.16
Filtrate.....	16.80	38.50	35.84
Amino N.....	0.64	1.90	1.90

## 1½ hr. digestion.

Residue.....	36.88	11.20	13.55
Filtrate.....	20.40	44.80	41.44
Amino N.....	0.70	2.40	2.40

## 2 hr. digestion.

Residue.....	35.93	10.36	11.20
Filtrate.....	22.65	48.44	46.90
Amino N.....	0.70	2.75	2.75

This series shows a uniformly better digestion in the cases in which no paracasein curd was formed. Whether the acid is added

first, followed by the pepsin, or whether both are added simultaneously apparently makes but little difference in the results obtained.

Series III having shown that the results obtained when acid and pepsin were added in the order named, or as a mixture, were practically identical, the procedure of adding a mixture was discontinued.

*Series IV.*

	1. cc.		2. cc.
Milk.....	10	Milk.....	10
H <sub>2</sub> O.....	10	H <sub>2</sub> O.....	10
Pepsin.....	5	1.2 per cent HCl.....	5
1.2 per cent HCl.....	5	Pepsin.....	5

Pepsin solution, 350 mg. in 100 cc.

1. Permitted clotting by pepsin before adding acid.

2. Added acid to precipitate casein before adding pepsin. No curd of paracasein was formed.

*Nitrogen.*

$\frac{1}{2}$  hr. digestion.

	1. mg.	2. mg.
Residue.....	40.80	26.46
Filtrate.....	17.92	31.36
Amino N.....	0.42	1.68

1 hr. digestion.

Residue.....	33.32	20.86
Filtrate.....	24.64	36.96
Amino N.....	1.40	2.24

$1\frac{1}{2}$  hr. digestion.

Residue.....	29.26	18.48
Filtrate.....	29.12	39.20
Amino N.....	1.68	2.80

2 hr. digestion.

Residue.....	26.18	15.54
Filtrate.....	31.92	42.28
Amino N.....	1.82	3.08

The relatively better digestion in the absence of a paracasein curd is shown here as in Series III.



## SUMMARY AND CONCLUSIONS.

1. Abderhalden's and Kramm's results, showing that clotted milk was more easily digested than milk in which coagulation had been prevented by the use of oxalate, were confirmed, but it is believed that the peptic digestion in the latter case was inhibited by the presence of the foreign oxalate solution.

It would appear that the internal digestion of the paracasein curd by adsorbed pepsin as set forth by Abderhalden and Kramm is of rather minor importance.

2. Using a slightly different method from that employed by Hawk, his conclusion is verified; namely, that pepsin exerts its action much more readily upon the protein constituents of milk in cases where no thick elastic curd of paracasein is formed.

3. The formation of a curd of paracasein depends upon two separate factors; *i.e.*, the strength of the HCl in the juice and the quantity of rennin present.

4. The peptic digestion of boiled milk is slightly more rapid than that of raw milk.

## THE PREPARATION OF GUANIDINE.\*

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(Received for publication, October 14, 1916.)

Levene and Senior<sup>1</sup> give a method used by them for the preparation of large quantities of guanidine. Their process, which is not new, seems satisfactory but the purity of the product when tested is not over 95 per cent.

For the past year we have been using a large amount of guanidine in various forms, and being unable to obtain any of the salts which usually come from Germany, we tried some of the various methods of preparation given in Beilstein's *Handbuch*, and adopted the following which not only gives a good yield but also a product of great purity. The method seems to have been first suggested by Delitsch and Volhard,<sup>2</sup> but they give no details. Guanidine may be obtained by heating dry ammonium thiocyanate in a short-necked Jena flask for 20 hours at a temperature from 190–200°C. For this purpose an iron or copper oil bath is necessary, using fairly heavy oil so as to ensure a temperature within the range stated.

### *Method.*

About 100 gm. of dry ammonium thiocyanate are placed in the flask and immersed in the oil, which should be above the level of the crystals. The heat is then raised gently until the required point is reached. When the 20 hours have expired, the mass is allowed to cool, extracted with hot water, and filtered. The filtrate, which contains the guanidine thiocyanate, is evaporated

\* The expenses connected with this work were defrayed by a grant from the Medical Research Committee.

<sup>1</sup> Levene, P. A., and Senior, J. K., *J. Biol. Chem.*, 1916, xxv, 623.

<sup>2</sup> Delitsch, G., *J. prakt. Chem.*, 1874, ix, 2. Volhard, J., *ibid.*, 1874, ix, 15.

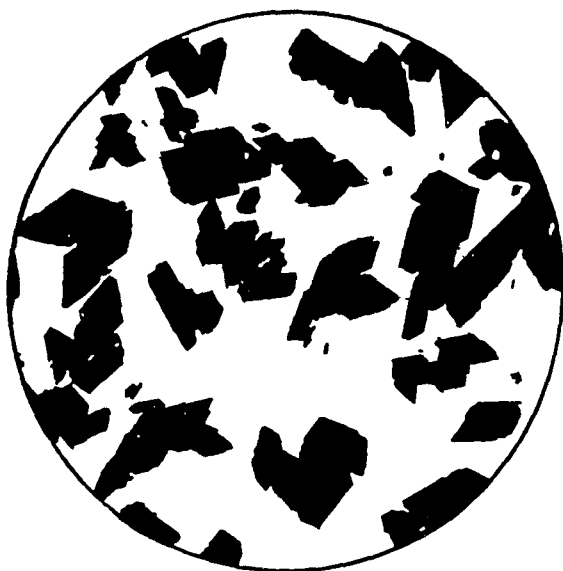


FIG. 1. Crystals of guanidine picrate. Microphotograph.  $\times 50$ .

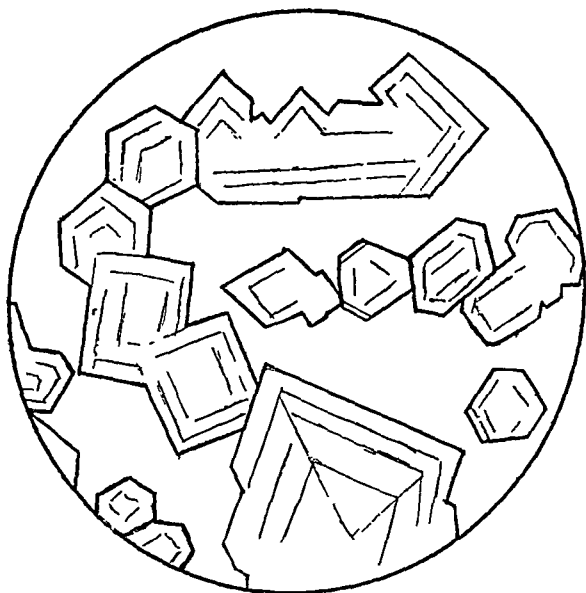


FIG. 2. Crystals of guanidine carbonate, produced by the process described. Camera lucida drawing.  $\times 20$ .

to dryness and weighed, and for every one part of the thiocyanate present, 0.58 part of potassium carbonate is added, with sufficient water for solution. The whole is then evaporated once more to dryness. The potassium thiocyanate is then removed by repeated treatment with 90 per cent ethyl alcohol, decanting off after each addition, until a drop of the supernatant liquid fails to give a red color with perchloride of iron. Crude guanidine carbonate is obtained by this means, as it is insoluble in alcohol. It can be further purified by dissolving in a small quantity of water and adding 90 per cent alcohol to about five or six times the original volume, and allowing to settle, when pure guanidine carbonate crystallizes out.

Free guanidine may be obtained from this salt by treatment with the theoretical quantity of dilute sulfuric acid and barium hydroxide, but for convenience in use and preservation, the carbonate is the best salt.

*Yield.*—100 gm. of ammonium thiocyanate yielded 15 to 20 gm. of pure guanidine carbonate.

### *Tests of Purity.*

1. Precipitation with sodium picrate: 0.1 gm. of prepared salt gave 0.158 gm. of picrate; theoretical 0.160 gm.

2. Nitrogen content of guanidine picrate formed.

	Calculated for	Found:	
	$\text{CH}_5\text{N}_3 \cdot \text{C}_6\text{H}_3\text{O}_7\text{N}_3$	a.	b.
N.....	29.11	29.50	28.77

3. Titration with 0.1 N sulfuric acid: 1 cc. 0.1 N  $\text{H}_2\text{SO}_4$  = 0.009 gm. guanidine carbonate. 0.18 gm. taken (indicator, methyl orange) required 20.01 cc. of acid; *i.e.*,  $20.01 \times 0.009 = 0.18009$  gm. guanidine carbonate.



FIG. 1. Crystals of guanidine picrate. Microphotograph.  $\times 50$ .

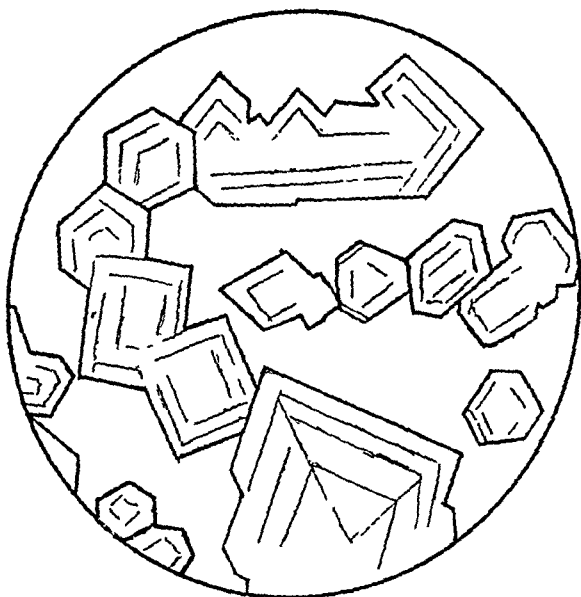


FIG. 2. Crystals of guanidine carbonate, produced by the process described. Camera lucida drawing.  $\times 20$ .

# PROTEINS OF THE CENTRAL NERVOUS SYSTEM.

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(Received for publication, November 6, 1916.)

## INTRODUCTION.

Although many workers have applied themselves to the elucidation of the chemistry of the nervous system, complete qualitative and quantitative studies of the proteins of the brain and nerves are lacking. This is due in great part to the impossibility of extracting from the fresh moist tissue more than a very small fraction of the total protein present. Even the examination of such extracted protein is attended with difficulties presented by the tenacity with which lipid substances and inorganic salts are held by the proteins, making the problem of purification a serious one. The ease with which protein substances undergo change in the presence of solvents used for their extraction or in process of separation and purification further involves the problem. At the basis of a complete qualitative and quantitative analysis of the proteins lies the necessity of separating the proximate constituents of the tissue. This must be performed in such a way that the properties of the proteins may be unchanged. The present research has for its object the elaboration of a method that would accomplish the isolation of the individual proteins, and the application of such a method to the investigation of the

\* At the time of his death Mr. McGregor was continuing certain phases of the investigation on the proteins of the brain. He had planned an elaborate study of his problem, involving not only a complete characterization of each protein but their amino-acid contents. In the development of this work he displayed unusual initiative; many of the points discovered were due to his independent study of them. In preparing his reports for publication his ideas and language have been scrupulously kept, though some of the matter reported is obviously preliminary.

C. G. MACARTHUR.

characteristics and relative amount of the proteins in the central nervous system.

The relations of the proteins to the functioning of the nerve cells is not understood. About 37 per cent of the dry substance of human brain tissue consists of proteins, and it is therefore to be anticipated that proteins play a relatively important part in the specific activities of the tissue. Scott<sup>1</sup> and Macallum,<sup>2</sup> in considering the nature of the Nissl substance of the nerve cells, regard it as axiomatic that an iron-holding nucleoprotein is necessary for the cell to carry on its normal function.

In considering the protein problem, the possibility was in mind that the different divisions of the human encephalon might be found to contain different proteins, and that the different species in the animal kingdom might exhibit characteristic differences in their brain proteins. Evidence of such modifications of substances has been gained by Reichert and Brown<sup>3</sup> in investigating the bloods of different animals.

The excellent method devised by Koch<sup>4</sup> for the estimation of the proximate constituents of animal tissues is the most efficient analytical scheme available for tissue work. It should be pointed out that this method is limited in its possible significance by our inadequate knowledge of the individual proteins. However, on the basis of the investigations of Halliburton,<sup>5</sup> Levene<sup>6</sup> and Ewald and Kühne,<sup>7</sup> on individual proteins Koch has divided the proteins into three fractions, simple proteins, nucleoprotein, and neurokeratin, the division in the case of the first two substances being accomplished by calculations based on the phosphorus content of the nucleoprotein. In the method of Koch the assumption is made that the proteins reported by Halliburton, together with the neurokeratin discovered by Ewald and Kühne, constitute all the proteins of the central nervous system. The

<sup>1</sup> Scott, F. H., *Tr. Canadian Institute*, 1899, vi, 405.

<sup>2</sup> Macallum, A. B., *Proc. Roy. Soc. Canada*, 1908, Series 3, ii, Section 4, 145.

<sup>3</sup> Reichert, E. T., and Brown, A. P., *The crystallography of hemoglobins*, Carnegie Institution of Washington, Publication No. 116, 1910.

<sup>4</sup> Koch, W., *J. Am. Chem. Soc.*, 1909, xxxi, 1329.

<sup>5</sup> Halliburton, W. D., *J. Physiol.*, 1894, xv, 90.

<sup>6</sup> Levene, P. A., *Arch. Neurol. Psychopath.*, 1899, ii, 1.

<sup>7</sup> Ewald, A., and Kühne, W., *Verhandl. naturh.-med. Vereins*, 1877, i, 457.

fact that by Halliburton's method less than 3 per cent of the total protein can be isolated, while neurokeratin represents about 15 per cent of the total proteins, makes it uncertain that the unextracted protein possesses the same characteristics as that actually separated. Yet our knowledge of the quantitative relations to the total proteins of so important a substance as nucleoprotein is based on two phosphorus determinations by Levene<sup>6</sup> on a nucleoprotein obtained from sheep brains by a modification of Halliburton's method. It was hoped that information gained from a complete protein analysis would furnish a basis for the extension of the method of Koch.

#### EXPERIMENTAL.

##### *The "Direct Solution" Method.*

In previous investigations on the proteins of the brain the value of the results is greatly minimized by the fact that the proteins are accounted for so incompletely. In the present investigation the first attempts to isolate the proteins were made by experimenting with the solvent action of various salt solutions on the moist sheep brain tissue. In so far as this tissue had been studied by chemical methods the results had shown great similarity to those from human brain tissue. On account of this fact, and also in view of the availability of absolutely fresh sheep brains, this tissue was adopted for preliminary study. M/10 solutions of the following substances were used as solvents:  $\text{NH}_4\text{Cl}$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{BaCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{MgSO}_4$ ,  $\text{K}_2\text{SO}_4$ ,  $\text{KAl}(\text{SO}_4)_2$ ,  $\text{KCl}$ ,  $\text{NaC}_2\text{H}_3\text{O}_2$ ,  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{CO}_3$ ,  $\text{NaCl}$ ,  $\text{NaOH}$ ,  $\text{Na}_2\text{B}_4\text{O}_7$ ,  $\text{NaKC}_4\text{H}_4\text{O}_6$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{Al}_2(\text{SO}_4)_3$ .

Following the method of Halliburton, 100 gm. of fresh tissue after mincing in a meat grinder were macerated for 24 hours at room temperature with 2 liters of the above solutions, thymol being added as an antiseptic. The turbid liquid was filtered through a Buchner funnel with the aid of filter paper pulp. Examination of the filtrates showed that in the case of the neutral or alkaline solvents, addition of weak acetic acid produced a precipitate, which is equivalent to Halliburton's "nucleoprotein." After filtering off this first precipitate, a second amount of protein was secured by boiling the solution. The markedly acidic solvents, like  $\text{ZnSO}_4$  and  $\text{KAl}(\text{SO}_4)_2$  yielded slight precipitates on neutralization, which were soluble in excess of dilute alkali or acid. Later experiments showed this material



to be an acid metaprotein produced by long contact of the tissue with the acid solutions. When solutions of weakly acid salts like  $\text{MgSO}_4$  were used, the protein not precipitated by acetic acid was found to be present in smaller quantity than in the solutions of neutral salts, and if the filtrates from the nucleoprotein were allowed to stand for 24 hours in contact with weak acetic acid, the second protein fraction in large part disappeared.

The quantity of protein extracted was estimated by nitrogen determinations on measured volumes of the clear filtrate:

1. N determination on first filtrate gives total N.
2. N determination on Solution 1 after precipitation with acetic acid: 1-2 gives nucleoprotein.
3. N determination on filtrate from 2, after boiling: 2-3 gives acid-soluble protein.

The following table shows that there are no very marked differences in the solvent properties of the individual neutral salts, the amount of protein extracted being about 1 per cent of the weight of the original moist tissue. The alkaline solvents extracted a larger quantity of protein.

*Nitrogen from 100 Gm. of Moist Tissue.*

Solvent.	Total N.	Nucleoprotein N.	Acid-soluble N.
	gm.	gm.	gm.
$\text{MgSO}_4$ .....	0.509	0.160	0.05
$\text{NaCl}$ .....	0.480	0.131	0.047
$\text{K}_2\text{SO}_4$ .....	0.558	0.165	0.043
$\text{KCl}$ .....	0.544	0.181	0.044
$\text{NaC}_2\text{H}_3\text{O}_2$ .....	0.450	0.101	0.056
$\text{NaHCO}_3$ .....	0.698	0.191	0.071
$\text{NaOH}$ .....	1.5	0.507	0.061
$\text{ZnSO}_4$ .....	0.262	0.0	0.034
$\text{KAl}(\text{SO}_4)_2$ .....	0.670	0.0	0.114

The above method of obtaining the proteins by direct extraction of the moist tissue was found to have many disadvantages that render it inapplicable to the quantitative study of nervous tissue proteins. These disadvantages may be briefly stated.

1. Small yield of protein.

2. Difficulty of filtration. This is one of the chief troubles encountered in the extraction of proteins, whether of plant or animal origin. Various means, such as that suggested by Os-

borne,<sup>8</sup> have been used to overcome it. The attempt was made to clarify the solution by means of aluminium cream, and by centrifuging, but so great a loss of protein occurred through occlusion or adsorption in the precipitate that these means were abandoned. Levene's<sup>6</sup> method of removing the suspended material by shaking with ether was not found to be very efficient.

3. Length of time required for extraction. 24 hours is required for extraction, and a similar period for precipitation and filtration. This gives opportunity for bacterial and hydrolytic changes.

4. The presence of lipoids in the precipitated protein. Halliburton reports lecithin as the most abundant impurity, while Levene states that "it is next to impossible even after continuous extraction with hot alcohol and ether during several weeks to get the product in such a condition that the evaporated alcohol or ether would leave absolutely no residue."

#### *The Method of Drying and Extraction of Lipoids.*

In view of the unsatisfactory results obtained by the above method of direct solution of the moist tissue, it was thought advisable to remove the lipoids as far as possible, in the hope that the proteins would thus be rendered more soluble, and the quantity of impurities minimized. This made it necessary to dry the tissue to prepare for the removal of lipoids by organic solvents. An objection that in the drying process similar changes might take place to those which vitiated the direct solution method was a serious one, and therefore to avoid all possibility of change in the tissue except that caused by loss of water, the following procedure was used.

Fresh sheep brains were ground in a meat chopper and spread in thin layers on large watch-glasses, then placed in a desiccator containing  $\text{CaCl}_2$ . The desiccator was surrounded by a freezing mixture. After the tissue was frozen the desiccator was continuously evacuated by a water pump for about 40 hours, by which time the tissue was found to be dry. It was ground in a mortar, then 20 gm. of the powder were treated with 300 cc. of benzene and the mixture was shaken in a glass-stoppered bottle on a mechanical shaking apparatus driven by a  $\frac{1}{2}$  horse power motor. After shaking for 3 hours, the yellow benzene extract was filtered off and

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<sup>8</sup> Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii,

a second and third extraction made. After three periods of agitation the residue was dried and weighed, the product being a nearly white powder which weighed 70 per cent of the weight of the original dry tissue. Further extraction with benzene yielded insignificant quantities of lipoids. Portions of the protein residue were shaken with water and with salt solutions, when a very great increase in solubility over the original moist tissue was noted, about 6 per cent of the original dry tissue being extracted as protein.

One of the difficulties inherent in the old method remained, however, in that the aqueous solutions contained much material in suspension and filtered very slowly. It was thought that a completer extraction of the lipoids would prevent this turbidity, and a systematic search was made for a solvent that would remove the maximum amount of lipid by cold extraction. By conducting the benzene extraction at a temperature of about 40° the percentage of lipid extracted was increased to about 40 per cent. Various mixtures of organic solvents, such as acetone, pyridine, or absolute alcohol, in benzene, were also found to be more efficient than pure benzene. The following table shows some of the solubilities of lipoids in the different solvents experimented with. Different samples of tissues were used in the various cases, but the general increase in solubility when alcohol is added to the benzene is shown clearly.

Solvent.	Dry tissue before extraction.	Residue after extraction.	Lipoid.
	gm.	gm.	per cent
Benzene.....	40.7	28.8	29.2
" .....	200	131.5	34.2
" .....	66.2	44.8	32.8
" .....	50	36.2	27.6
" at 40°.....	20	12.25	38.75
" " 40°.....	63.5	39.34	38.05
" 10 per cent acetone.....	20	13.85	30.9
" 10 " " aniline.....	20	14.45	27.75
" 5 " " alcohol.....	20	11.20	44
" 5 " " " .....	20	11.49	42.55
" 5 " " " .....	20	11.00	45
" 15 " " " .....	20	11.36	43.2
" 25 " " " .....	20	11.25	43.8

Thus a 5 per cent solution of absolute alcohol in benzene is found to extract in 10 hours about 45 per cent of the dry tissue.

That this solvent does not interfere with the solubility of the proteins is shown by the following experiment.

Three similar samples of air-dried sheep brain were extracted with different solvents, (1) benzene, (2) equal volumes of benzene and acetone, (3) 5 per cent absolute alcohol in benzene. The residues after complete extraction were shaken with solutions of 2 per cent NaCl, equal volumes of the filtrate were coagulated by heat, and the protein coagula weighed. The following table summarizes the result.

Lipoid solvent.	Dry tissue.	Tissue after lipid extraction.	Lipoid extracted.	Protein extracted by NaCl. Dry tissue.
	gm.	gm.	per cent	per cent
Benzene.....	20	13.52	32.4	5.04
Benzene-acetone.....	20	13.25	33.7	5.25
Benzene-alcohol.....	20	10.23	48.8	5.04

The benzene-alcohol mixture, although extracting a much larger quantity of lipid, does not cause a greater amount of protein to become soluble. It may be supposed that the 5 per cent protein obtained represents the amount of protein in the tissue that is soluble in neutral solvents. This point will be further considered when the quantitative relations of the proteins are discussed. A solution of 5 per cent absolute alcohol in benzene was adopted for removal of lipoids, and will be termed the "lipoid solvent."

The difficulty of filtering the aqueous extracts was noticed to increase when the temperature of the solvent water approached the temperature of the room. Recourse was made to cooled water for use in extraction, and with this expedient a nearly clear solution was obtained upon extracting the tissue, which filtered very readily through a Buchner funnel, using ordinary filter paper.

By the use of the freezing method above described it is possible to work with only small amounts of material. This method is free from objection as far as its effect on the tissue is concerned and may therefore be used as a check on the air-drying method here described for preparing large quantities of tissue.

To dry large quantities, hashed brain tissue after addition of 0.1 per cent thymol was spread in thin layers on large glass plates, which were

placed on shelves in a drying chamber through which air was drawn by an electric fan. By turning the tissue frequently so as to prevent film formation, drying can be accomplished in 20 hours, or in less time, depending upon the thickness of the layers of tissue. The material can then be powdered and desiccated over  $\text{CaCl}_2$  *in vacuo* to remove the last traces of moisture. To compare the solubility of the proteins obtained by the freezing and air-drying methods, hashed sheep brain was divided into two portions, one of which was dried by the freezing method, the other in the air chamber. The dried tissue was then thoroughly extracted with the lipid solvent. 3 gm. portions of the vacuum-dried and air-dried material were shaken with 300 cc. of 2 per cent  $\text{NaCl}$ , and two 50 cc. portions of each of the clear filtrates were taken for total protein determination.

	Protein in 50 cc.	
	Vacuum-dried.	Air-dried.
	gm.	gm.
Precipitated by excess alcohol.....	0.0820	0.0830
Coagulated by addition of acid at boiling point..	0.0743	0.0737

This established the fact that air-drying does not affect the protein of the tissue to any appreciable extent.

#### *Preparation of Tissue by Lipoid Extraction Method.*

Through arrangement with Armour and Company, Chicago, sheep brains from animals killed on the day of shipment were packed in ice and expressed to this laboratory, 24 hours being consumed in transit. The tissue was in excellent condition, and was free from blood except that contained in the pia mater. Lessem and Gies<sup>9</sup> used sheep brain tissue received 24 hours after the death of the animals, and report that no appreciable changes in the tissue could be shown to have taken place in this short period.

To test further this question of possible change in the tissue, the brain of a dog removed from the skull 7 hours after the animal was killed was sectioned along the longitudinal fissure, and one-half dried immediately in the air dryer, the tissue drying in 3 hours. The remaining half of the brain was left for 3 days in a refrigerator, then dried in the usual way. Each half was extracted separately with benzene, and 2 gm. portions of

<sup>9</sup> Lessem, W. W., and Gies, W. J., *Am. J. Physiol.*, 1902-03, viii, 183.

the residue extracted with 300 cc. of 10 per cent  $\text{MgSO}_4$ . Nitrogen determinations on equal volumes of the filtrates before and after coagulation of the proteins were made, to compare the amount of protein in the two extracts. The results are as follows:

	Fresh tissue.	Stale tissue.
	gm.	gm.
Weight before drying.....	28	28
“ after “ .....	5.9	5.9
“ “ benzene extraction.....	4.1	4.1
Nitrogen in 25 cc. before boiling.....	0.0077	0.0079
“ after boiling.....	0.0041	0.0043
Protein N in 25 cc.....	0.0036	0.0036

This shows that if the tissue is carefully preserved in a cold place very little change takes place in the solubility of the protein.

The brains as received from Chicago were sectioned into three portions, cerebrum, cerebellum, and the medulla with the pons and optic lobes. The tissue from the cerebrum was used in all the following experiments, except where otherwise stated. A very simple and effective means was used to remove the pia mater with blood vessels. A single cerebrum was placed in a mortar and gently tapped with a pestle until the membrane was loosened. The entire membrane could then be picked up with forceps and removed. Occasionally portions of the more closely adherent membranes remained, but these small amounts were removed by sieving the tissue after drying. The membrane- and blood-free tissue was passed through a meat grinder, mixed thoroughly with 0.1 per cent thymol, and spread in thin layers on glass plates which were placed in the drying chamber. After drying, the tissue was shaken with the lipid solvent (1 liter to 100 gm.), filtered through cheese-cloth, dried from benzene in the air dryer, ground in a coffee mill, and passed through a 20-mesh sieve. Small amounts of membrane which had not been removed from the original tissue were retained on the sieve. The powdery product was next extracted for two 3 hour periods with the lipid solvent, when a yellow, nearly odorless powder resulted on drying, which yielded only traces of lipoids when further treated with the solvent. As is usually found with dry protein powders, the product was extremely hygroscopic. If allowed to remain exposed to the air of the laboratory it rapidly took up water, gaining about 10 per cent in weight without at all losing its powdery appearance. Such moisture-containing protein slowly decomposed, as could be recognized by the development of slight odor and the change of solubility shown by the proteins. The protein powder thus prepared was kept in a vacuum desiccator over  $\text{CaCl}_2$  and can thus be preserved for months without apparent change in its properties.

*General Method of Studying the Proteins.*

Various groups of proteins may be differentiated from others by differences in their solubilities. By the use of different solvents, it was proposed to divide the proteins into groups, then investigate the character of the protein or proteins in each group. The solvents used were water, aqueous solutions of neutral salts, acid solutions, alkaline solutions, and alcohol. On account of the finding by Ulpiani and Lelli<sup>10</sup> of a nuclein extracted by chloroform, extracts were also made with this solvent.

*Proteins Extracted by Neutral Solvents.*

Solutions of the protein powder were made in 2 per cent NaCl, 2 per cent Na<sub>2</sub>SO<sub>4</sub>, 10 per cent MgSO<sub>4</sub>, and in distilled water. The solvent was first cooled to about 10°, then shaken with the powder for ½ hour on the mechanical shaker. The resulting solution was strained through cloth, and the nearly clear liquid could then be filtered through ordinary filter paper in a Buchner funnel in about ½ hour. The importance of letting the tissue stand for as short a time as possible in contact with the solvent was shown by extracting 12 gm. of the protein powder with 1,500 cc. of distilled water. Three 100 cc. portions of the filtrate were taken, the first portion coagulated by heat, and the protein weighed. To Portion 2 were added 10 gm. of MgSO<sub>4</sub>, and the solution placed, together with Portion 3, in a refrigerator, and left 48 hours. The protein was then estimated by heat coagulation, and in each case less protein was found in the solutions which had been allowed to stand.

	Protein. gm.
Portion 1. Aqueous solution.....	0.0816
“ 2. MgSO <sub>4</sub> “ .....	0.0735
“ 3. Aqueous “ .....	0.0807

It is seen that the presence of the slightly acid salt MgSO<sub>4</sub> accelerates the decomposition of the protein.

The solutions of the proteins prepared as above described were studied by precipitation of the protein with weak acetic acid total heat coagulation, fractional heat coagulation, fractions salting out, precipitation by alcohol, and by dialysis.

a. *Precipitation by Acids.*—It had been observed by Halliburton<sup>5</sup> that from solutions of brain proteins a precipitate is thrown down upon addition of weak acetic acid. In the case of salt

<sup>10</sup> Ulpiani, C., and Lelli, G., *Gazz. chim. ital.*, 1902, xxxii, 466.

extracts, he showed the presence of protein material in solution after the precipitation with acetic acid, but when distilled water was used as a solvent, no such protein was found. These results were confirmed in the present work in using the dried and lipid-extracted tissue, with this important exception, that the distilled water solution held protein in solution after acetic acid precipitation. That is, the behavior of the distilled water solution was quite similar to that of the salt extract. The minimum concentration of acetic acid required to produce precipitation in solutions of lipid-extracted tissue was 0.05 per cent for 2 per cent NaCl solutions, and 0.02 per cent for distilled water. The higher concentration which Halliburton found it necessary to use, 0.15 per cent, is perhaps attributable to the protective action of the lipoids present.

In comparing the ratio of protein precipitated by acetic acid to that left in solution when different solvents were used, it was found that the ratios depend on the concentration of acid used and on the medium in which precipitated.

This fact was observed by making a distilled water solution of the protein powder, 30 gm. to 1,000 cc. of water. The clear filtrate was divided into four equal parts. Part 1 was coagulated by heating to boiling in the presence of a trace of acid, and the total protein weighed. To Part 2, NaCl was added to make a 2 per cent solution; to Part 3,  $(\text{NH}_4)_2\text{SO}_4$  was added to 10 per cent concentration, and Part 4 was left without salt addition. Each of Solutions 2, 3, and 4 was divided into two equal parts and different amounts of acetic acid were added. The precipitates were filtered off and weighed, and the protein in the filtrate coagulated and weighed. The following table shows the weights of the two fractions obtained, expressed in terms of gm. of protein from equal volumes of original solution.

Solution.	Concentrated $\text{HC}_2\text{H}_3\text{O}_2$ .	Protein precipitated.	Protein in filtrate.	Total.
	gm.	gm.	gm.	gm.
1. Water.....				0.1045
2. 2 per cent NaCl.....	0.2	0.0615	0.0163	0.0778
	0.05	0.0544	0.0317	0.0861
3. 10 per cent $(\text{NH}_4)_2\text{SO}_4$ .....	1	0.0912	0.00	0.0912
	0.05	0.0516	0.0431	0.0907
4. Water.....	0.02	0.0376	0.0620	0.0996
	0.1	0.00	0.0951	0.0951



It is apparent from the above table that no constant protein fraction is split off by acetic acid, and it is further significant that after treatment with acid the total protein is not recovered in the two fractions. The fact above noted as an exception to the finding of Halliburton, that distilled water holds a portion of the protein in solution after acid treatment, may here receive an explanation. In the older method, the acid used to precipitate the so called nucleoprotein was allowed to remain in the solution in contact with the soluble protein, and hydrolysis changed this protein over to metaprotein. This assumption has been tested on the lipoid-extracted tissue by allowing a solution from which a precipitate had been obtained by acetic acid to stand for a day, when it was found that a large proportion of the soluble protein had been changed to metaprotein and was precipitated on neutralization. It is evident therefore that the acid-soluble protein varies in amount according to the means taken to obtain it. The fact that it is soluble in distilled water is proof that it is not in any sense a true globulin.

*b. Total Heat Coagulation.*—The changes produced in the protein by small concentration of acid or change of medium are paralleled by changes produced by heat coagulation from different solvents.

MgSO<sub>4</sub>, NaCl, and distilled water extracts were made and the protein coagulated by addition at the boiling point of sufficient acetic acid to make the concentration 0.01 per cent, this being the minimum concentration at which complete agglutination and precipitation would take place. Phosphorus determinations on the precipitated protein were made. Some of the values obtained from protein coagulated from different solvents are as follows:

*Phosphorus.*

H <sub>2</sub> O	NaCl	MgSO <sub>4</sub>
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.1557	0.0537	0.1538
0.1718	0.0756	
0.1395	0.0725	
0.1793	0.0663	
	0.0833	
	0.0560	

It is seen that the phosphorus content of the protein depends upon the method of isolating it from solution.

*c. Fractional Heat Coagulation.*—A 2 per cent  $\text{Na}_2\text{SO}_4$  solution of the protein was made, to which were added 2 cc. of 1 per cent lactic acid per 100 cc., to agglutinate the protein when coagulation occurred. Five 25 cc. portions of the solution were placed in a water bath at an initial temperature of  $45^\circ$ . After 5 minutes Tube 1 was withdrawn and immediately placed in ice to cool. The temperature of the bath was raised to  $50^\circ$  for 5 minutes, at which point Tube 2 was removed. The heating was continued for 5 minute periods at the temperatures noted in the table below. After cooling, the precipitates were filtered off and weighed.

No. of 5 min. periods heated.	Temperature.	Weight of coagulum.
	$^\circ\text{C}.$	<i>gm.</i>
1	45	0.0170
2	50	0.0280
3	55	0.0360
4	60	0.0440
5	70	0.0510

Another experiment using a solution of different concentration, where the heating intervals were 10 minutes, is noted.

No. of 10 min. periods heated.	Temperature.	Weight of coagulum.
	$^\circ\text{C}.$	<i>gm.</i>
1	42.5	
2	45	0.0100
3	47.5	0.0215
4	50	0.0280
5	52.5	0.0300
6	55	0.0340
7	57.5	0.0350
8	60	0.0385
9	62.5	0.0400

Similar experiments have been repeated a number of times with substantially the same result, showing that a progressive coagulation takes place, no temperatures being noted at which excessive coagulation occurred which would point to the presence of a protein differing from the remainder of the solution. The phenomenon here observed is in accordance with the work of

Chick and Martin<sup>11</sup> who have shown that the heat coagulation of proteins is a reaction the velocity of which varies considerably with different proteins, and according to the acidity or salinity of the solution. It would appear from the data given above that the protein in solution is an individual and not a mixture. If the data of Halliburton are examined in the light of such studies on velocity of coagulation, the separation of three proteins by means of fractional heat coagulation may be given this same interpretation; i.e., that the protein is a simple one and not three. The details of the method for fractional coagulation are not reported in Halliburton's paper describing the brain proteins; but in a previous study of serum albumin and serum globulin<sup>12</sup> a method is described which presumably was followed in the case of nervous tissue.

The solution to be coagulated was made slightly acid by the addition of 1 cc. of 2 per cent acetic acid to each 100 cc. "With regard to the length of time which a liquid has to be kept at any given temperature to ensure complete precipitation of the proteid at that temperature, it has been found that 5 minutes is as a rule sufficient." It is obvious from the above data and from the work of Chick and Martin that at any temperature a precipitate may be obtained, and if 5 minutes is given for a precipitate to form, the division into three proteins is easily explicable. The presence of considerable quantities of lipoids may also affect the speed and temperature of coagulation. This does not, however, furnish an explanation of the finding by Halliburton of only traces of phosphorus and nuclein in the fractions coagulated at 45° and 75°, while the 56° coagulating protein was rich in phosphorus and nuclein. Halliburton's data on the presence of nuclein are as follows:

Coagulation temperature.	Nuclein after gastric digestion.
°C.	per cent
47	2.13
56	5.26
75	Imponderable.

d. *Fractional Salting Out.*—Fractional salting out with  $(\text{NH}_4)_2\text{SO}_4$  was done using solutions of proteins made with distilled water and with 2 per cent NaCl.

<sup>11</sup> Chick, H., and Martin, C. J., *J. Physiol.*, 1910, xl, 404.

<sup>12</sup> Halliburton, *J. Physiol.*, 1884, v, 152.

Five 200 cc. portions of the clear filtrates from each of these extracts were placed in 500 cc. measuring flasks, to which different amounts of a solution of 50 gm. of  $(\text{NH}_4)_2\text{SO}_4$  in 100 cc. of solution were added. The solutions were then made up to 500 cc. and allowed to precipitate. 150 cc. portions were filtered off, and the protein was coagulated by heat; these coagula therefore represent the amounts of protein remaining in solution. The weights are given below under I. To 250 cc. portions filtered from each measuring flask 10 cc. of 1 per cent acetic acid were added. The weights of precipitates thus obtained are shown in Column II. 150 cc. of the filtrate from II were coagulated by heat; the weight of coagulum is recorded in Column III. Column IV shows the sum of II and III. The weights of the various fractions are calculated, for ease of comparison, on the basis of the total volume, 500 cc.

Solution.	$(\text{NH}_4)_2\text{SO}_4$ to 500 cc.	I. Total.	II. Acid- insoluble.	III. Acid- soluble.	IV.
	cc.	gm.	gm.	gm.	gm.
Distilled water.....	100	0.3362	0.1856	0.1042	0.2898
	150	0.2926	0.1476	0.1026	0.2502
	200	0.1866	0.0710	0.0878	0.1588
	300	0.1144	0.0280	0.0476	0.0756
	500	0.0536		0.0274	0.0274
2 per cent NaCl.....	100	0.5770	0.3082	0.1888	0.4970
	150	0.4960	0.2756	0.1832	0.4588
	200	0.3640	0.1862	0.1586	0.3448
	250	0.2280	0.0940	0.0978	0.1918
	500	0.1056	0.0320	0.0510	0.0830

It will be seen from Column I that this precipitation is of a similar additive nature to the fractional heat coagulation process. Columns II and III show increased amounts of protein precipitated with rising concentrations of  $(\text{NH}_4)_2\text{SO}_4$ , and confirm the conclusion from the data given on page 414, that on treatment with acid the total protein is not recovered in the two fractions. The ratios of precipitates shown by Columns II and III are not, indeed, constant, but the steady decrease of both sets of quantities with increased concentration of salt points to the probable presence of a unit substance before precipitation; the disparity between the ratios may be explained in the light of the results shown in the table on page 413, the ratio being in part dependent on the medium from which the precipitate is obtained. These results may be explained on the assumption that the protein extracted by water and by neutral salts is an individual and not a mixture, and that on treatment with acetic acid a cleavage

takes place, yielding a soluble and an insoluble protein product, while a third part of the original protein is not precipitated on boiling the solution.

Qualitative fractional salting out experiments with anhydrous  $\text{Na}_2\text{SO}_4$  at  $30^\circ$ , where the relative volumes of the precipitates were noted, gave the same results as the above, the precipitates gradually increasing with rising concentration of  $\text{Na}_2\text{SO}_4$ . The results of two experiments in which  $\text{NaCl}$  extracts were salted out with  $(\text{NH}_4)_2\text{SO}_4$  are given below. In Experiment I, cerebral tissue from beef brains, treated similarly to sheep tissue, was used. 40 cc. samples of the extracts were taken, to which were added different amounts of 50 per cent solution of  $(\text{NH}_4)_2\text{SO}_4$ , then made up to 100 cc. After precipitation, equal volumes of the filtrates were coagulated, and the weight of protein obtained.

Experiment I.		Experiment II.	
50 per cent $(\text{NH}_4)_2\text{SO}_4$ in 100 cc.	Weight of protein in filtrate.	50 per cent $(\text{NH}_4)_2\text{SO}_4$ in 100 cc.	Weight of protein in filtrate.
cc.	gm.	cc.	gm.
25	0.0399	30	0.0550
27.5	0.0377	35	0.0490
30	0.0356	40	0.0440
35	0.0307	45	0.0354
40	0.0240	50	0.0250
45	0.0182	55	0.0204

Two criticisms of the conclusions of Halliburton emerge from these data: first, that fractional salting out does not necessarily yield different products, as was assumed from the fact that  $\text{MgSO}_4$  yielded precipitates at different concentrations. It is to be noted that no very definite line separated the proteins obtained by Halliburton by fractional salting out. The three fractions separated out at the following concentrations.

	Concentration of $\text{MgSO}_4$ at:	
	Commencement of precipitation	Completion of precipitation.
	per cent	per cent
Fraction 1.....	30	50
" 2.....	50	90
" 3.....	80	100

The second objection lies in the effect observed when varying concentrations of acid are used, inconstant amounts of protein being precipitated. On this account it is impossible to assume that the protein precipitated represents a definite substance which existed preformed in the tissue and is wholly precipitated by weak acids. Levene<sup>6</sup> showed that the nucleoprotein obtained by him was extremely unstable: it rapidly lost its initial solubility in dilute alkalis and could therefore not be easily purified by this method. A small quantity which was dissolved in weak alkali and reprecipitated, immediately after the first precipitation with acetic acid, showed a phosphorus content of 0.45 per cent as compared with 0.56 per cent before such treatment.

*e. Precipitation by Alcohol.*—The differences exhibited by the proteins when obtained by different methods suggested the possibility of getting the protein intact by avoiding the use of heat or acid in separating it from solution. It was found that two volumes of alcohol added to one of the distilled water extract would cause ready separation of the protein. On determining the phosphorus content of such precipitates, a marked increase was found over the protein prepared in other ways, the phosphorus content varying from 0.7 to 1.2 per cent in different samples. Very little of this phosphorus could be removed by extraction with hot ether and alcohol, showing that the excess phosphorus was not due to phosphatides. By repeated extraction of the alcohol-precipitated protein with water, however, the phosphorus content was reduced to 0.11 per cent. Other analyses gave values between 0.11 and 0.14 per cent. The water contained phosphates directly precipitable by molybdate solution, but yielded a greater amount of phosphorus to molybdate solution after oxidation with nitric and sulfuric acids, showing that part of the phosphorus was in organic combination. A portion of the water extract was evaporated to dryness; the residue contained, in addition to inorganic salts, a small amount of organic material which was not again soluble in water, and which gave weak responses to protein color tests. The action of alcohol on the tissues apparently produces decomposition, and, further, results in the adsorption or occlusion of large amounts of phosphates. The protein after precipitation by alcohol could not be redissolved, so attempts were made to dialyze the phosphates and other impurities from the protein solution.

*f. Dialysis.*—500 cc. of the extract were dialyzed in a parchment vessel for 2 days against running water, a small quantity of thymol being added as a preservative. The protein was then precipitated by  $(\text{NH}_4)_2\text{SO}_4$ , when phosphorus determination showed a content of 0.718 per cent.

Part of this material was also tested for the presence of iron. Reference has been made earlier in this paper to the finding by histologists<sup>1</sup> that the Nissl bodies in nerve cells contain iron, apparently in protein combination. There is no record in the literature of iron being detected in the proteins after separation and purification. To test the protein for this element, small amounts were decomposed (1) by incineration in a porcelain crucible and (2) by oxidation with nitric acid. Control experiments were run, which gave negative results when tested with  $\text{K}_4\text{Fe}(\text{CN})_6$  and  $\text{KCNS}$ . The solutions obtained from the decomposed protein, however, yielded unmistakable precipitates of Prussian blue and red coloration with thiocyanate. The test for iron has been applied to the protein separated by coagulation and by salting out, and has always given a positive reaction.

### *Proteins Extracted by Acidic Solvents.*

Acid extractions of the lipid-extracted tissue were made with 1 per cent acetic acid and with 5 per cent solutions of oxalic, citric, and boric acids. The clear filtrate in each of these cases yielded a precipitate when neutralized. That the protein thus obtained represents a decomposition product of true tissue protein was made evident by completely extracting some of the dry tissue with 2 per cent  $\text{NaCl}$  solution, then shaking the residue with the solvents above noted. In no case did any precipitate appear on neutralizing these acid filtrates. Further, if to the 2 per cent  $\text{NaCl}$  solution 1 per cent of acetic acid be added and the solution shaken, a precipitate appears on neutralizing. Reference is made on page 414 to the slow formation of meta-protein by permitting the distilled water solution of the protein to stand in contact with weak acetic acid. This explains the appearance of the "new protein" obtained by Marie<sup>13</sup> by compressing fresh brain tissue to which had been added 1 per cent

<sup>13</sup> Marie, A., *Compt. rend. Soc. biol.*, 1911, lxx, 322, 459; 1911, lxxi, 709; 1912, lxxii, 100, 528.

of acetic acid, and for which he proposed the name "*antilyssine*." It may be stated that such an acid metaprotein does not exist preformed in the brain.

*Proteins Extracted by Alkaline Solvents.*

Tissue which has been extracted as completely as possible with dilute salt solutions or distilled water invariably yields protein to a weak ammonia solution. This had been pointed out by Levene, in the direct extraction of moist tissue. Levene considered this protein as identical with the nucleoprotein extracted by dilute salt solutions. After thorough extraction with ammonia up to 1 per cent concentration, a further amount of protein is taken up by 0.5 per cent NaOH. The method of Schkarin<sup>14</sup> for the extraction of "neurostromin" is based on this fact. The protein extracted by  $\text{NH}_4\text{OH}$  or by NaOH is not precipitated upon neutralization, but rapidly deposits from solution when the acid concentration reaches 0.1 per cent acetic. No protein remains in solution after the precipitation by acid, in distinction from the nucleoprotein obtained from water extracts. Analysis of the protein after purification by resolution and reprecipitation shows a phosphorus content of about 0.6 per cent. A slight though definite reaction for iron is given by the purified substance. The protein is readily soluble in very dilute alkalies and does not lose this property even after continued standing in the presence of acetic acid. In this it differs from the nucleoprotein that is soluble in distilled water.

*Extraction of the Tissue with Alcohol.*

In order to settle the question of a protein similar to the prolamine group being present in nervous tissue, 10 gm. of the dried material were shaken with 300 cc. of 70 per cent alcohol. A slight residue on evaporation of the filtered alcohol gave negative reactions to all protein color tests applied.

<sup>14</sup> Schkarin, A. N., Inaugural Dissertation, St. Petersburg, 1902, i, 453.



*Extraction of the Tissue with Chloroform.*

Extraction of the dry tissue with chloroform was carried out in view of the finding of Ulpiani and Lelli,<sup>10</sup> confirmed by Steel and Gies<sup>15</sup> that the chloroform-water extract of fresh tissue contains nuclein. 30 gm. of dried tissue, before extraction with the lipoid solvent, was treated with 150 cc. of chloroform and shaken for  $\frac{1}{2}$  hour. To the yellow chloroform extract an equal volume of ethyl acetate was added, which produced immediately a pure white waxy precipitate. This precipitate was tested for protein in various ways, but all the tests, including Millon's and biuret which Steel and Gies had found positive, were negative.

*Quantitative Relations of the Proteins.*

It has been shown above that water and weak alkalies extract proteins from dried and lipoid-extracted nervous tissue, and from the means taken to obtain these substances it may be assumed that they exist preformed in the tissue and are not cleavage products produced by the action of the solvents. It has also been shown that acidic solvents do not extract unchanged proteins. The quantitative relations of the proteins remain to be considered.

Air-dried tissue from the cerebrum of the sheep brain, after thorough desiccation *in vacuo*, was quantitatively extracted with the lipoid solvent. The extracted material was ground in a mortar, then sieved through fine cloth until a nearly impalpable powder resulted. This was carefully dried *in vacuo* and a weighed sample taken for analysis. The ease with which the protein forms a sticky, unfilterable emulsion with solvents at room temperatures makes it imperative to work with solutions at about 10° when repeated extractions are to be made. In the following extractions, ice water was used in each case, and it was found that the solutions filtered readily after shaking for  $\frac{1}{2}$  hour. Three extractions of the weighed sample were made with distilled water, the residue being quantitatively recovered after each extraction. The protein in the extract was estimated by coagulating a measured volume in the presence of 0.02 per cent acetic acid, and weighing. By evaporating to dryness a measured volume of the extract, the weight of the total extracted material was obtained, and by difference the non-protein fraction calculated. 0.05 per cent  $\text{NH}_4\text{OH}$  was used to extract the residue from the third water extraction, and the

<sup>15</sup> Steel, M., and Gies, W. J., *Am. J. Physiol.*, 1907-08, xx, 378.

protein in this solution was weighed after precipitation by addition of 10 cc. of 1 per cent acetic acid to each 100 cc. of extract. The residue from  $\text{NH}_4\text{OH}$  was first treated with 0.05 per cent  $\text{NaOH}$  and the protein estimated in the same way as from the  $\text{NH}_4\text{OH}$  solution. The final residue was dried in the drying chamber, ground to a fine powder, and extracted with 0.1 per cent and then with 1.0 per cent  $\text{NaOH}$ , neither of which solvents removed any further protein. The following table shows the result of the analysis.

Solvent.	Original dry tissue	
	Protein.	Non-protein.
	<i>per cent</i>	<i>per cent</i>
Benzene-alcohol .....		45.92
Water, 1st extraction .....	4.17	10.1
"    2nd    "    .....	0.539	0.1
"    3rd    "    .....	0.0	0.0
0.05 per cent $\text{NH}_4\text{OH}$ .....	3.753	1.12
0.05 "    " $\text{NaOH}$ .....	5.906	6.32
	14.368	63.56
	63.56	
	77.928	

The residue from these extractions was treated with hot alcohol under a reflux condenser for 24 hours, but no residue was obtained on evaporation. It was then oxidized with  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  for phosphorus determination. 0.284 per cent phosphorus was found. The presence of phosphorus in the residue after complete extraction with lipid and protein solvents is capable of several interpretations. First, it may be due to the presence of an insoluble phosphorus-containing protein. Second, it may be found that neurokeratin itself contains phosphorus, and, as suggested in the previous discussion of neurokeratin, the substance described by Ewald and Kühne may be a product resulting from digestive action on a phosphorus-containing protein which constitutes the true supporting tissue. Again, the protein may represent an unextracted protein which has become insoluble as a result of the processes to which the tissue has been subjected.

### *Proteins from Different Divisions of Sheep Brain.*

It has been stated that the brain tissue used in most of the experiments described in this paper was prepared from the cerebrum of the sheep. Qualitative tests were made to determine

whether the different divisions of the brain of this animal contain similar proteins.

Four divisions of the brain were examined separately. The cerebrum was divided into two parts by section across the cruciate sulcus; also the cerebellum was separated from the medulla with pons and optic lobes. These four divisions were dried, extracted with the lipoid solvent, and the dry material was shaken with water and with salt solutions. Examination of the solutions showed the presence of a protein that yielded a precipitate with acetic acid and left a quantity of protein in solution which was coagulated on boiling. Thus the properties were similar to those exhibited by the protein from the whole cerebrum. By extraction with weak alkalis a second yield of protein was obtained, which possessed the same characteristics, as regards stability towards acids and absence of splitting products, as the alkali-soluble protein from the cerebrum.

### *Proteins from Nervous Tissue of Different Animals.*

The brains of several animals other than the sheep have been examined qualitatively with respect to the proteins present, and will be considered separately.

*Beef Brain.*—Experiment I, page 418, shows the fractional precipitation by  $(\text{NH}_4)_2\text{SO}_4$  of a saline extract of beef brain, where exactly the same phenomena are observed as in the case of sheep tissue, and it may therefore be assumed that the protein of the beef brain soluble in neutral solvents is an individual substance. The distilled water extract of beef brain also gives a precipitate with dilute acetic acid, and yields more protein on boiling the filtrate. That protein soluble in dilute alkalis is present was shown by completely extracting the tissue with 2 per cent NaCl and subsequently shaking with dilute  $\text{NH}_4\text{OH}$ . The filtrate, on slightly acidifying with acetic acid, deposited a protein having the same general properties as the corresponding protein from the sheep brain.

*Dog Brain.*—Data are given on page 411 on the extraction of the proteins from the brain of the dog. This tissue was tested in a similar manner to beef brain. The distilled water extract yielded a soluble and an insoluble fraction on addition of acetic acid. Dilute alkalis extracted from the tissue a protein corresponding to that of the sheep brain.

*Rabbit Brain.*—Fresh brain obtained within a few minutes after the death of the rabbit has been analyzed for neutral salt-

soluble protein, and the result found positive. In the case of this tissue no subsequent extraction was made with dilute alkalies.

*Human Brain.*—A portion of the cerebrum from human brain tissue received about 48 hours after death was dried and the lipoids were extracted in the same way as from sheep brains.

20 gm. of the air-dried tissue extracted thoroughly with 5 per cent absolute alcohol in benzene left a residue of 11.49 gm., or 57.45 per cent of the dry tissue. The lipid-extracted material was shaken with 2 per cent NaCl. Weak acetic acid was added to a portion of the filtrate, when protein material rapidly precipitated, and upon boiling the filtrate from this precipitate a further amount of protein was obtained.

This corresponds exactly with the properties of the protein from sheep brain. After complete extraction with NaCl solutions, the human brain yielded protein to dilute alkalies, precipitable upon acidifying.

#### DISCUSSION OF RESULTS.

The activity of the central nervous system presents such a complexity of reactions, involving the structure and intercommunications of ten billions of cells, and the constitution and properties of all the substances within the cells, that the relation of a single group of substances like the proteins to the integrated action of the tissue will be learned only through patient and persistent work on the problem.

The properties, however, of isolated constituents may furnish a clue to their functions in the tissues. It is from this standpoint, in part, that the results here presented are to be considered. It is found that a protein substance of extreme lability is present, the properties of which show it to be especially reactive to slight changes in acidity or alkalinity. The transmission of the nervous impulse along a fiber has been shown by Tashiro<sup>16</sup> to be accompanied by the production of CO<sub>2</sub>, and we may therefore assume that the change in the nerve cell body which results from the entrance of the impulse might be initiated by a change in the H ion concentration in the cell substance. In the nucleoprotein described we may see, in the absence of more conclusive data,

<sup>16</sup> Tashiro, S., *Am. J. Physiol.*, 1913, xxxii, 107.

a substance possessing the instability requisite for quick response to such mild stimuli.

The fact that histological evidence points to the presence of two phosphorus-containing proteins in the nerve cells, may possibly be related to the isolation of two such proteins, one substance being soluble in neutral media, the other requiring very small concentration of alkali for its solution.

It is remarkable that the proteins obtained from the brains of various animals should be so similar in their properties as to give identical qualitative reactions. The same conclusion has been reached with regard to the inorganic constituents of brain tissue by Weil,<sup>17</sup> through a complete study of the ash of the human brain and that of the ox. He concludes that the psychical differences in the two species are due to different ratios of gray and white substance. In the case of the proteins, the absolute identity of the proteins in the different animals has not been shown; the significance of the quantitative method outlined in this paper lies in its application to the preparation of the various proteins, to permit of precise characterization.

#### SUMMARY.

I. A new method of preparing nervous tissue for quantitative study of the constituents, especially proteins, is described. The soluble proteins can be directly and completely extracted by suitable solvents, and quantitatively estimated.

II. The proteins of the central nervous system are at least three in number:

1. A phosphorus- and iron-containing protein soluble in distilled water. It comprises about 5 per cent of the dry brain tissue. The properties of this protein may be summarized in the following statements. (a) The protein extracted from brain tissue by neutral salts or by distilled water is precipitated in a progressive, additive manner by increasing concentrations of  $(\text{NH}_4)_2\text{SO}_4$ . (b) Fractional heat coagulation of such solutions shows a similar additive coagulation of the protein. (c) These data point to the individual nature of the protein. (d) The protein whether extracted by distilled water or by neutral salt

<sup>17</sup> Weil, A., *Z. physiol. Chem.*, 1914, lxxxix, 349.

solutions shows the same general properties. No true globulin can be said to be present. (e) The protein is very unstable. In the presence of low concentrations of acid it breaks down, yielding at least three products, the natures of which are dependent on the concentration of acid used and on the medium in which the products are precipitated. (f) The protein when obtained by total heat coagulation varies in character according to the solvent from which it is coagulated, and the amount of acid used to effect coagulation. (g) The protein obtained by different methods has always given a slight though definite positive reaction when tested for the presence of iron. (h) The phosphorus content is about 0.11 per cent.

2. A phosphorus- and iron-containing protein soluble in dilute alkalis. Approximately 10 per cent of the dry tissue is present in this form. (a) The protein extracted by alkaline solvents is qualitatively and quantitatively different from that extracted by neutral solvents. (b) The protein extracted by alkaline solvents may be completely precipitated by the addition of acid, and the protein is not decomposed in the precipitation. (c) This protein contains 0.60 per cent of phosphorus.

3. Supporting tissue, insoluble in neutral, acidic, or alkaline solvents. This makes up about 20 per cent of the original dry brain tissue.

III. The brains of the ox, rabbit, dog, sheep, and man contain, in general, similar proteins.

IV. The different divisions of the sheep brain contain similar proteins.

V. The central nervous system does not contain preformed the "globulins" described by Halliburton.<sup>5</sup>

VI. Acid metaprotein, described by Marie,<sup>13</sup> does not exist preformed in the brain.



# A NOTE ON THE INFLUENCE OF INOSITE UPON THE EXCRETION OF PHENOL IN THE DOG.

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As a result of his work on the utilization of inosite, Anderson<sup>1</sup> found that in the dog, after feeding a known amount of inosite, he could recover in the feces up to 77 per cent of the amount, while the urine contained only minimal amounts. In man<sup>2</sup> 9 per cent was recovered in the urine while none at all could be demonstrated in the feces. The question as to what happens to the rest of the inosite is left open.

In accordance with the belief that inosite is a hexahydroxybenzene<sup>3</sup> and that inosite undergoes bacterial fermentation in the intestine it seems plausible to consider the possibility that the benzene fraction may be split off, thus giving rise to phenol.<sup>4</sup> Consequently it was thought worth while to try to demonstrate an increase in the phenol content of the urine after feeding inosite.<sup>5</sup>

The results of such feeding are shown in the table, and it is evident that no "extra" phenol appears in the urine upon the ingestion of inosite, so that the rôle which this substance plays in metabolism is still in doubt.

<sup>1</sup> Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

<sup>2</sup> Anderson, R. J., and Bosworth, A. W., *J. Biol. Chem.*, 1916, xxv, 399.

<sup>3</sup> Hammarsten, O., and Hedin, S. G., *Physiological Chemistry*, New York, 7th edition, 1915, 579.

<sup>4</sup> Folin O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.

<sup>5</sup> The inosite was obtained through the kindness of Dr. Anderson.



*Influence of Inosite on the Excretion of Phenol in the Dog.*

Date.	Total N.	Phenols.				Inosite.			
		Free.	Total.	Free.	Conjugated.	Fed.	Recovered.		
							Urine.	Feces.	Percentage of amount fed.
1918		gm.	gm.	per cent	per cent	gm.	gm.	gm.	
Oct. 15	14.82	0.203	0.267	76.2	23.8				
" 16	14.89	0.200	0.270	74.2	25.8				
" 17	14.99	0.208	0.277	75.2	24.8	5.0	None.		
" 18	14.75	0.203	0.272	74.8	25.2			1.875*	37.5
" 19	14.81	0.202	0.267	75.8	24.2	20.0	0.114		
" 20	15.00	0.203	0.270	75.3	24.7			8.234†	41.2

\* Feces of 17th and 18th.

† Feces of 19th and 20th.

As for the actual feeding and recovery of inosite, the results obtained corroborate those of Anderson.<sup>1</sup>

## STUDIES OF INFANT FEEDING.

### THE CHEMICAL CHANGES PRODUCED BY THE ADDITION OF LIME WATER TO MILK.

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While the addition of lime water to milk used as infant's food has been common practice for many years, it has not been known just what changes the lime water produces, and as far as we know the subject has not been investigated from a purely chemical standpoint. The use of lime water in this connection has been based, first, upon the erroneous assumption that the acidity of cow's milk is much higher than that of human milk<sup>1</sup> and must be reduced before it can be used as a food for infants; and, second, upon the well known fact that lime water when added to milk in sufficient amount inhibits the curdling of the casein by rennin and hence, by deduction, will prevent the formation of casein curds in the stomach, a source of much trouble in infant feeding. As the addition of any alkali to milk causes a precipitation of insoluble calcium phosphate, a fact well known to those familiar with the chemistry of milk, and in view of the results recently obtained by us with reference to the availability of dicalcium phosphate when present as a constituent of infants' food, it seemed desirable that more definite information should be obtained concerning the chemical changes produced by the addition of lime water to milk.

The technique employed in this investigation is the same as that used by Van Slyke and Bosworth<sup>2</sup> in their studies of cow's

<sup>1</sup> Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 707.

<sup>2</sup> Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 135. Bosworth, *ibid.*, 1915, xx, 707. Bosworth and Van Slyke, *ibid.*, 1916, xxiv, 177.

milk, human milk, and goat's milk. A complete chemical analysis of the milk being first obtained, it is then filtered through porous porcelain filters. The filtration divides it into two portions, a soluble filterable portion and an insoluble or unfilterable portion.

TABLE I.

*Changes Produced by the Addition of Lime Water to Cow's Milk.*

Sample No.....	1		2			
Lime water added, per cent.....	0	50	0	16½	33½	50
Reaction (+ = acidity; - = alkalinity) as cc. of 0.1 N required to neutralize 100 cc.						
Whole milk.....	+6.8	-15.3	+8.4	0.0	-7.0	-16.6
Serum.....	+6.6	+ 1.9	+7.8	+4.2	+2.2	+ 2.2
CaO*	gm.	gm.	gm.	gm.	gm.	gm.
Total.....	0.1947	0.2982	0.2106	0.2505	0.2710	0.3141
Insoluble combined with casein.....	0.0759	0.0759	0.0857	0.0857	0.0857	0.0857
Insoluble combined with P <sub>2</sub> O <sub>5</sub> .....	0.0524	0.1750	0.0586	0.0934	0.1291	0.1738
Total insoluble.....	0.1283	0.2509	0.1443	0.1891	0.2148	0.2595
Soluble.....	0.0664	0.0473	0.0663	0.0614	0.0562	0.0546
P <sub>2</sub> O <sub>5</sub> *						
Total.....	0.2314	0.2314	0.2554	0.2554	0.2554	0.2554
Organic in casein.....	0.0497	0.0497	0.0545	0.0545	0.0545	0.0545
Inorganic, total.....	0.1817	0.1817	0.2009	0.2009	0.2009	0.2009
“ insoluble.....	0.0633	0.1433	0.0752	0.1172	0.1376	0.1595
“ soluble.....	0.1154	0.0284	0.1257	0.0837	0.0633	0.0414
Citric acid.*						
Total.....	0.1684	0.1684	0.1795	0.1795	0.1795	0.1795
Insoluble.....	0.0000	0.0381	0.0000	0.0000	0.0184	0.0370
Soluble.....	0.1684	0.1301	0.1795	0.1795	0.1611	0.1425
Casein.....	3.10	3.10	3.40	3.40	3.40	3.40

\* Per 100 cc. of original milk.

The composition of these two portions was determined by chemical analysis.

Cow's milk when used as a food for infants is usually diluted with an equal or greater volume of water which may carry other

substances in solution or suspension, such as lactose, calcium hydroxide, barley flour, maltose, etc., and in order to make the conditions surrounding the milk used for this investigation as nearly comparable to feeding conditions as possible the desired amount of lime water was added to the milk, and this mixture then diluted to twice the volume of the original milk. The results obtained by our study, however, have been reported on the basis of the original volume of the milk.

Our investigation shows that while the addition of lime water to milk increases the total CaO present it brings about a marked change in the arrangement of the salts, which results in a pre-

TABLE II.

*Insoluble Acids and Bases in Milk and Milk Plus Lime Water Calculated to Gm. Equivalents.*

Sample No.	Lime water added.	Casein as an octavalent acid.	$\frac{P_2O_5}{2}$ as a divalent acid.	Citric acid as trivalent acid.	Sum of acids.	Calcium.	Excess of base.
	per cent						
1	0	$27.9 \times 10^{-4}$	$17.8 \times 10^{-4}$	$0.0 \times 10^{-4}$	$45.7 \times 10^{-4}$	$45.8 \times 10^{-4}$	$0.1 \times 10^{-4}$
1	50	27.9	40.4	6.0	74.3	89.6	15.3
2	0	30.6	21.2	0.0	51.8	51.5	0.0
2	16 $\frac{2}{3}$	30.6	33.0	0.0	63.6	67.5	3.9
2	33 $\frac{1}{3}$	30.6	38.8	2.9	72.3	76.7	4.4
2	50	30.6	44.9	5.8	81.3	92.7	11.4

cipitation of calcium, phosphorus, and citric acid. The figures in Table I show that by the addition to milk of 50 per cent of lime water the soluble CaO is reduced to less than one-half of the amount present in the original milk, the soluble phosphorus to less than a third, and the soluble citric acid to about three-fourths.

The amounts of the insoluble constituents present in the milk and the milk to which the lime water has been added as shown in Table I have been calculated to gm. equivalents and will be found in Table II. In making these calculations phosphoric acid has been considered a divalent acid because it has been shown that the insoluble inorganic phosphorus normally present in fresh cow's milk is in the form of dicalcium phosphate,  $CaHPO_4$ .<sup>3</sup> It

<sup>3</sup> Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 135.

will be noticed that in the fresh milk there is a balance of bases and acids, while the addition of lime water to the milk produces an excess of base, which means of course, that the insoluble phosphate is now a mixture of di- and tricalcium phosphate.

The acidity or alkalinity as determined by the method of Van Slyke and Bosworth<sup>4</sup> also demonstrates the presence of alkaline phosphates, for it will be noticed that while the original milk and its serum had an acid reaction, the addition of lime water brought the reaction of the serum towards the neutral point and the reaction of the unfiltered milk became alkaline, showing that in filtering the milk to obtain the serum, insoluble alkaline salts were removed or failed to pass through the filter. If milk to which 50 per cent of lime water has been added is centrifugalized, a sediment is obtained which is a mixture of calcium caseinate and calcium phosphates. Such a sediment obtained by us and treated with alcohol and ether to dry it gave the following figures upon analysis.

	per cent
Casein.....	21.84
Total $P_2O_5$ .....	34.784
Organic $P_2O_5$ in casein.....	0.350
Inorganic $P_2O_5$ .....	34.434
CaO.....	32.474

Upon calculating these figures to gm. equivalents, we obtain casein  $19.7 \times 10^{-3}$ ,  $\frac{P_2O_5}{2}$  inorganic as a divalent acid  $969.8 \times 10^{-3}$ , sum of casein and inorganic  $P_2O_5$   $989.5 \times 10^{-3}$ , calcium oxide  $1,159.8 \times 10^{-3}$ . It will be seen from these figures that the calcium present is more than enough to form the neutral dicalcium phosphate with the  $P_2O_5$  present, but not enough to form the alkaline tricalcium phosphate, the result being a mixture of the two.

In this connection it is of interest to recall that Clark<sup>5</sup> in studying the change in hydrogen ion concentration produced by the addition of lime water, or sodium citrate to milk concluded that the use of sodium citrate was the more objectionable because it produced an alkaline reaction in the milk, while the use of

<sup>4</sup> Van Slyke and Bosworth, *J. Biol. Chem.*, 1914, xix, 73.

<sup>5</sup> Clark, W. M., *J. Med. Research*, 1914-15, xxxi. 431.

lime water produces a reaction near the neutral point. As he used the electrical method to determine his hydrogen ion concentrations it will be seen at once that he failed to take into consideration the insoluble alkaline phosphates present.

#### CONCLUSIONS.

The addition of lime water to milk, which normally contains some insoluble dicalcium phosphate, results in the precipitation of more calcium phosphate, the insoluble phosphates under these conditions being a mixture of di- and tricalcium phosphate,  $\text{CaHPO}_4$  and  $\text{Ca}_3\text{P}_2\text{O}_8$ .

The addition of lime water to milk brings the reaction of the serum towards the neutral point, the soluble alkalinity of the lime water being used up in the precipitation of the insoluble calcium phosphates mentioned above.

When milk to be used for infant feeding is treated with lime water and finally diluted to such an extent that it has twice the volume of the original milk, or more, the soluble calcium and phosphorus may be reduced to amounts less than those which are present in human milk.

On hydrolysis, the saponin yielded a crystalline sapogenin melting at  $175^{\circ}\text{C}.$ , also glucose and apparently glucuronic acid.

We have not been able to isolate any saponin from *Yucca filamentosa* which agrees with that reported by Schulz. It is possible that saponins from the same species of plants may differ, depending on the place where grown and the time of year when picked. We have some evidence on this point from work done recently on other species of plants.

#### EXPERIMENTAL.

*Preparation of the Saponin.*—The rootstocks of *Yucca filamentosa* were cut into slices and dried at  $50^{\circ}\text{C}.$  in air desiccated over calcium chloride. The dried substance was ground to a coarse powder in a mill. 5 kilos of the substance were extracted five times with hot 95 per cent alcohol by heating in a steam kettle. The alcoholic extract was concentrated to a small volume in a vacuum still, 200 gm. of magnesium oxide were added, and the mixture was evaporated to dryness on the steam bath. The residue was pulverized and extracted with hot absolute alcohol. On standing, the saponin was deposited as a light brown amorphous substance. This was filtered off and washed with absolute ether, the yield of crude saponin being about 6 per cent. It was purified by dissolving in hot absolute alcohol, cooling the solution, filtering, and finally washing the saponin with absolute ether. To obtain the saponin colorless and free from ash, it was necessary to repeat this process several times. On pouring the mother liquors into an equal volume of absolute ether, more saponin was obtained. The supernatant liquid was decanted and the precipitates were purified as stated above.

The aqueous solution of the saponin was neutral to litmus and had the foaming properties characteristic of most saponins. Lead acetate, lead subacetate, and barium hydroxide did not precipitate it from the dilute or concentrated aqueous solution. A cholesterol compound could not be made. When evaporated with sulfuric acid it gave a deep red color. The saponin was very hygroscopic and, for analysis, was first dried in a vacuum oven by gradually heating to  $105^{\circ}\text{C}.$  It was then dried over night in an electric oven at  $110^{\circ}\text{C}.$

Analyses of four different preparations gave the following results.

I.	0.2009 gm.	substance	gave	0.3838 gm.	$\text{CO}_2$	and	0.1295 gm.	$\text{H}_2\text{O}.$
II.	0.2471	"	"	0.4731	"	"	0.1602	"
III.	0.1962	"	"	0.3749	"	"	0.1245	"
IV.	0.2608	"	"	0.4992	"	"	0.1657	"

	I.	II.	III.	IV.	Average.
C.....	52.10	52.21	52.12	52.20	52.16
H.....	7.21	7.25	7.10	7.11	7.17
O.....	40.69	40.54	40.78	40.69	40.67

0.3609 gm. substance lowered the freezing point of 34.29 gm. of phenol 0.147°.

$$M = \frac{7,500 \times 0.3609}{34.29 \times 0.147} = 537$$

	Calculated for $C_{21}H_{40}O_{11}$	Found:
C.....	52.17	52.16
H.....	7.25	7.17
Mol. wt.....	552	537

*Hemolytic Action and Surface Tension of the Saponin.*<sup>6</sup>—Blood was drawn from the heart of a Belgian hare into 0.9 per cent salt solution, containing a little potassium oxalate. The mixture was then centrifugated and was washed several times with Locke's<sup>7</sup> solution. Two drops of the residual mass of corpuscles were then added to 10 cc. of the aqueous solution of the saponin, 1 in 20,000, and this was kept at 37°C. Hemolysis took place in about 15 minutes. For the surface tension determination, 100 mg. of the saponin were dissolved in 1 liter of Locke's solution. The surface tension at 37°C., as determined by the Morgan drop weight method, was 56.69 dynes per cm.

#### *Hydrolysis of the Saponin.*

*Preparation of the Sapogenin.*—10 gm. of the saponin were dissolved in 100 cc. of 4 per cent sulfuric acid and the solution was heated for 7 hours on the steam bath. A tan-colored amorphous substance separated. The mixture was centrifugated and the substance washed with water until no longer acid to litmus. It was then dried over the steam bath and placed over sulfuric acid in a vacuum desiccator, where it became red and

<sup>6</sup> These determinations were made by Dr. H. E. Woodward of the Bureau of Chemistry.

<sup>7</sup> Locke's solution:

	gm.
NaCl.....	9.29
KCl.....	0.42
CaCl <sub>2</sub> .....	0.24
NaHCO <sub>3</sub> .....	0.15
H <sub>2</sub> O to 1 liter.	



horny in appearance. The yield was 4.9 gm., or about 50 per cent.

To complete the hydrolysis this sapogenin was then boiled with 50 cc. of 6 per cent sulfuric acid for 7 hours. At first it became soft and oily, but after about 5 hours it changed to a dark hard lump. The sulfuric acid then was poured off and the mass ground up and washed with water till the washings were no longer acid to litmus. The solid material dissolved completely in acetone and was easily crystallized from dilute acetone or dilute alcohol. The fine, acicular crystals melted at 175°C. The alcoholic solution of this sapogenin was not precipitated by alcoholic lead acetate or basic lead acetate. No change was observed on adding an alcoholic solution of ferric chloride. It was insoluble in 10 per cent alkali and acids. Glacial acetic acid dissolved it readily.

The molecular weight determined in phenol was 255. 0.1004 gm. of substance lowered the freezing point of 26.17 gm. of phenol 0.113°.

$$M = \frac{7,500 \times 0.1004}{26.17 \times 0.113} = 255$$

*Identification of Glucose.*—The acid solutions obtained by the hydrolysis of the saponin were neutralized with barium carbonate, filtered, clarified with animal charcoal, and evaporated on the steam bath, thus giving a yellow syrup. On cooling, this syrup solidified. We obtained a phenyl osazone melting at 205°C. and a bromophenyl osazone melting at 220°C. We did not succeed in preparing a hydrazone, indicating the absence of mannose. The absence of mannose, together with the dextrorotation of the aqueous solution of the syrup, and the melting points of the osazones show that this sugar is glucose.

*Tests Indicating the Presence of Glucuronic Acid.*—In addition to glucose, the sugar syrup obtained by hydrolysis of the saponin also contained a substance which gave furfural on distillation with 12.5 per cent hydrochloric acid. A pentose phenyl or bromophenyl hydrazone or osazone could not be prepared, and Bial's<sup>3</sup> reagent (orcin, hydrochloric acid, and a trace of ferric chloride) failed to give a positive test. It is therefore unlikely that a pentose was present.

<sup>3</sup> Bial, M., *Biochem. Z.*, 1907, iii, 323.

When the syrup was heated with naphthoresorcinol and hydrochloric acid, and the cooled mixture shaken with ether, the ether solution was colored a beautiful purple. The reaction with naphthoresorcinol and the formation of furfural together with the failure of Bial's reagent to give a positive test are strong indications of the presence of glucuronic acid. The close resemblance of the phenyl and bromophenyl osazone of glucose and glucuronic acid makes it difficult to identify the latter in a mixture of the two.

The presence of glucuronic acid as a product of hydrolysis in substances obtained from plants has been reported in a few cases, and Asahina and Momoya<sup>9</sup> have established its presence in the saponin of *Styrax japonica*.

Lack of material prevented further work.

#### *Location of the Saponin in the Tissue.*

The saponin has been definitely located in the fibrovascular bundles of the roots and the leaf bases. Since the plant belongs to the monocotyledons, a great number of fibrovascular bundles occur in the roots. The number of bundles roughly estimated in a root (1 cm. thick) was considerably over 1,000. The saponin was found within the tracheæ. In almost every bundle a trachea cell was observed, which was partially or wholly filled with an amorphous mass, more or less transparent. The color of these masses was orange-brown in the roots and brownish red in the bases of the leaves. Saponin masses were not found in parenchyma cells of the wood or bark. This observation is especially interesting since, as far as a study of the literature shows, this is the first time that saponin has been found in the tracheæ.

The coloration in the tracheæ obtained with sulfuric acid was first distinctly yellow, followed by a very slow change to pink and reddish violet. After standing several hours the color became dark brown and the tissue was largely destroyed. Occasionally deep brownish red globule-like bodies were observed in or close to the tracheæ.

When acetic anhydride was added to the section before the

<sup>9</sup> Asahina, Y., and Momoya, M., *Arch. pharm.*, 1914, cclii, 56.

addition of sulfuric acid or sulfuric acid-alcohol, the color change to pink or reddish violet could be observed almost immediately. Slight heating of the section with sulfuric acid-alcohol also had the same effect in the coloring of the tracheæ bundles. Addition of a trace of 10 per cent ferric chloride solution to the section freshly mounted in sulfuric acid-alcohol produced a decided green color. Precipitates were not observed.

Since experiments with the isolated saponin had shown that it could not be precipitated by neutral or basic lead acetate, barium hydroxide, or cholesterol, it was thought probable that the hemolytic properties of the saponin might be made use of in supporting the previous evidence as to the location of the saponin in the tissue. For this purpose blood from the heart of a rabbit was obtained and, after defibrinizing or adding sodium oxalate to prevent the precipitation of fibrin, the blood corpuscles were washed with physiological sodium chloride and Locke's solution. The blood corpuscles obtained by centrifugating were directly applied in a suspension of about 1:1 Locke's solution to the dry section. The turbid suspension infiltrated into the tissue which became invisible under the microscope, but in a few seconds certain parts of the tissue cleared up. The blood corpuscles disappeared, the liquid became transparent and discolored, and the tissue clearly visible. Repeated observations showed that the tracheæ cells always were visible first and the masses in these consequently had the strongest hemolytic action. Crystals of the saponin obtained as previously described were tested with blood in the same manner under the microscope, but practically no hemolytic action was observed.

Briefly stated, the saponin masses present in the tracheæ had the same properties as the saponin isolated from the plant. They were easily soluble in water and also in hot alcohol (95 per cent), and insoluble in benzene or chloroform. They showed the characteristic color reactions with concentrated sulfuric acid, sulfuric acid-alcohol (1:1), sulfuric acid with the addition of a trace of a 10 per cent ferric chloride solution, or acetic anhydride followed by concentrated sulfuric acid. The masses had a strong hemolytic action.

The microchemical experiments were carried out on fresh material collected in the fall, on the same material dried at room

temperature, and also on the same material dried for several hours at 100°C. Cross and longitudinal sections were prepared and treated either directly with the reagents or after extraction with ether to remove fatty and coloring substances.

While in material dried at 100°C. the saponin masses covered the cell walls of the tracheæ like a film, often not easily visible, these masses were very conspicuous in material dried at room temperature.

#### SUMMARY.

1. A new saponin,  $C_{24}H_{40}O_{14}$ , was isolated from the rootstock of *Yucca filamentosa*. The yield of crude saponin was about 6 per cent. It was soluble in water, alcohol, phenol, and glacial acetic acid, and could not be precipitated from the aqueous solution by neutral lead acetate, basic lead acetate, and barium hydroxide. No cholesterol compound could be prepared. Hemolysis was observed after 15 minutes in the saponin solution (1 to 20,000) containing rabbit blood, and kept at 37°C. The surface tension at 37°C. was 56.69 dynes per cm.

2. Hydrolysis yielded a sapogenin, crystallizing in regular, fine needles melting at 175°C. The molecular weight of this sapogenin was approximately 255. It was soluble in alcohol, phenol, and glacial acetic acid, but was not soluble in either alkali or dilute acids. Alcoholic solutions of lead acetate, basic lead acetate, and ferric chloride had no effect on the alcoholic solution of this substance. The crystals tested under the microscope had practically no hemolytic action on blood corpuscles.

3. Hydrolysis of this saponin also yielded a sugar identified as glucose. Evidence was obtained which indicates that glucuronic acid is formed when the saponin is hydrolyzed.

4. The saponin is located as brownish amorphous masses in the fibrovascular bundles of the roots and leaf bases.



# A NEW METHOD OF STEAM DISTILLATION FOR THE DETERMINATION OF THE VOLATILE FATTY ACIDS, INCLUDING A SERIES OF COLORIMETRIC QUALITATIVE REACTIONS FOR THEIR IDENTIFICATION.\*

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## INTRODUCTION.

In the course of an investigation in this laboratory it became necessary to have at hand a simple and convenient method for the identification and quantitative determination of the volatile saturated fatty acids, and to that end the investigation here reported was undertaken.

The imperfections of the well known Duclaux method for the determination of these acids are generally recognized among chemists who have occasion to apply it. When mixtures of acids are distilled identification by the distillation numbers alone is somewhat uncertain, a fact rather lightly touched upon

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by Duclaux himself.<sup>1</sup> Calculating results from the distillation numbers is a tedious process and, such being the case, appreciable errors may be introduced.

The method about to be described for the identification and determination of the volatile fatty acids is one of steam distillation rather than direct distillation. By the use of this method of steam distillation the vaporization rate of the individual acids, when plotted on a simple logarithmic chart, appear as straight lines. On the other hand, the vaporization rate of mixtures of acids will be depicted on the same chart as curved lines. For purposes of identification, therefore, the results of the steam distillation may be interpreted by direct comparison with the chart instead of with tables as in the Duclaux method. In addition, there is given a series of colorimetric qualitative tests, depending upon the difference in solubility of the iron and copper salts of the acids in various organic solvents, which serve to confirm the results of the steam distillation. The calculations in the method herein described are of the simplest nature and are doubtless familiar to every chemist.

Attention is called to an article by Stein<sup>2</sup> in 1913, which seems to have passed unnoticed as far as can be judged from the recent literature. Stein states: If dilute aqueous solutions of substances volatile with steam are distilled under conditions of constant pressure, temperature, and volume in the distilling flask, the process proceeds with a certain regularity. Stein gives the credit for this discovery to Naumann and Müller.<sup>3</sup>

Stein carried out distillations with twenty organic acids, seven of which consisted of the first members of the volatile fatty acids. In most cases he made these distillations while keeping the volume of the liquid in the distilling flask at 150 cc. He constructed a table giving the percentage amount of each acid distilling over into the first 100 cc. fraction of the

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<sup>1</sup> Duclaux, " . . . en cherchant sur les tables si la marche des nombres trouvés par l'expérience coïncide avec une des séries calculées. Cette coïncidence n'est naturellement jamais parfaite: les incertitudes de la méthode des distillations, les irrégularités inévitables dans la mesure des prises et dans les dosages ne le permettent pas." Duclaux, *Dosage des principaux produits de la fermentation alcoolique, Traité de Micro-biologie*, 1900, iii, 394.

<sup>2</sup> Stein, A., *Gesetzmässigkeiten bei der Wasserdampfdestillation organischer Säuren*, *J. prakt. Chem.*, 1913, lxxxviii, 83.

<sup>3</sup> Naumann, A., and Müller, W., *Regelmässigkeiten beim Destilliren verdünnter wässriger Phenollösungen*, *Ber. chem. Ges.*, 1901, xxxiv, 224.

distillate while the volume in the distilling flask was maintained at 150 cc. His results are recorded in Table I.

Some of the percentages obtained by Stein from duplicate determinations made on the same acid do not appear to agree very closely. There is no description of the steam distilling apparatus used in determining these percentages and it is possible that the one made use of was somewhat unsuited to the purpose. The distillations were, therefore, repeated in this laboratory, the same acids being used to redetermine the percentage amounts. In addition, there have been added to the list the percentage amounts of acid distilling over into the first 100 cc. fraction for isocaproic, heptylic, caprylic, and pelargonic acids. Of the three remaining higher acids, capric acid is soluble only to a very slight extent, undecylic acid was not available, and lauric acid is practically insoluble. Therefore these three acids were not included in this investigation. The figures obtained with the acids investigated will be found in Table I under those obtained by Stein.

TABLE I.

*Comparative Percentages of Acid Which Distil Over into the First 100 Cc. Fraction of Distillate from a Constant Volume of 150 Cc. as Found by Stein and by Dyer.*

Acid.	Formic.	Acetic.	Propionic.	n-Butyric.	Iso-butyric.	Valeric.**	n-Caproic.	Iso-caproic.	Heptylic.	Caprylic.	Pelargonic.
Stein.	20.98 22.02	33.09	55.21 58.51	70.53	79.3 80.35 80.85	87.7	91 94				
Dyer.	17.89*	30.75*	52.67*	69.88	79.73	87.51	89.75	92.60	94.52	98.45	100.00

\* Average of determinations as noted below in Table II.

\*\* Stein's valeric acid was evidently the iso acid since it had the same distilling constant as the isovaleric acid used in this laboratory.

The figures for the first three acids mentioned represent the average percentages from several determinations as shown in Table II. The values found for formic, acetic, and propionic acids are somewhat lower than those obtained by Stein; in the other cases the figures agree fairly well.

The results so obtained indicated that a practical use might be made of the "distilling constants" for the identification and determination of those fatty acids volatile with steam. As already noted, the second 100 cc. fraction of the distillate had



# 448 Determination of Volatile Fatty Acids

TABLE II.

*Amount of Each Acid Taken for the Distillation Together with the Amount of That Acid Distilling Over into the First and (in Some Cases) Second 100 Cc. Fractions of Distillate as Determined by Titration with 0.1 N KOH.*

Acid.	Total amount of acid distilled.	Amount of acid present in 1st 100 cc. fraction.		Amount of acid present in 2nd 100 cc. fraction.		Calculated amount of acid in 2nd 100 cc. fraction.†
	gm.	gm.	per cent	gm.	per cent	per cent
Formic.						
Sample 1*...	0.49413	0.08882	17.97	0.07732	15.65	14.69
“ 2*...	0.59873	0.10585	17.68	0.08974	14.99	14.55
“ 2....	0.58962	0.10623	18.01	0.09411	15.96	14.77
Average.....			17.89		15.53	14.67
Acetic.						
Sample 1*...	0.22192	0.06821	30.74	0.04863	21.91	21.29
“ 1....	0.41008**	0.12573	30.66			
“ 1....	0.41075**	0.13362	30.74			
“ 1....	0.41111	0.12621	30.70			
“ 1....	0.41267†	0.12849	31.14			
“ 1....	0.62708	0.19496	31.09	0.13696	21.86	21.04
“ 2*...	0.41033	0.12447	30.34	0.08868	21.61	21.13
“ 2....	0.41333	0.12609	30.51	0.09162	22.16	21.20
“ 3*...	0.41891	0.12915	30.83	0.09186	21.17	21.33
Average.....			30.75		21.74	21.20
Propionic.						
Sample 1*...	0.47637	0.24974	52.43	0.11850	24.88	24.94
“ 1....	0.48622•	0.25774	53.01	0.12332	25.36	24.91
“ 1....	0.98220	0.51629	52.56	0.23708	24.14	24.93
Average.....			52.67		24.79	24.93

\*Sample 1 of formic acid, Eimer and Amend, "from Germany."

" 2 " " Baker and Adamson, "100 per cent."

" 1 " acetic " old sample of glacial acid, source unknown.

" 2 " " J. T. Baker, "99.5 per cent."

" 3 " " source unknown, "95.00 per cent."

" 1 " propionic acid, Kahlbaum.

\*\* Distillate collected in 10 cc. fractions.

† Distillate collected in an ordinary 100 cc. graduate. Other distillates were collected in various 100 cc. graduated flasks.

‡ The figures here given were obtained as described under Experimental.

TABLE III.  
*Titrations. Cc. of 0.1 N KOH Required for Each Successive 10 Cc. Fraction of Distillate up to 100 Cc.*

Acid..... Cc. of 0.1 N KOH..... Equivalent gm. of acid.	Formic. 216.05 0.9943	Acetic. 171.52 1.0298	Propionic. 132.64 0.9822	n-Butyric. 106.52 0.9383	Isobutyric. 107.19 0.9443	Isovaleric. 91.45 0.9338	n-Caproic. 8.19 0.0951	Isocaproic. 8.93 0.1037	Heptylic. 14.26 0.1856	Caprylic. 6.44 0.0928	Pelargonic. 2.82 0.0446
cc.											
1	4.40	6.11	9.33	12.22	16.74	17.40	1.85	1.92	3.43	2.18	0.80
2	4.28	6.04	8.70	10.88	13.88	13.78	1.35	1.56	2.67	1.27	0.60
3	4.30	5.87	8.12	9.44	12.20	11.32	1.00	1.20	2.12	0.78	0.38
4	4.20	5.56	7.53	8.78	10.37	9.10	0.82	0.98	1.60	0.65	0.22
5	4.18	5.44	7.09	7.48	8.48	7.60	0.62	0.76	1.28	0.50	0.20
6	4.17	5.13	6.64	6.52	7.46	6.08	0.49	0.56	0.77	0.30	0.10
7	4.07	4.94	6.23	5.65	6.13	5.10	0.40	0.43	0.55	0.20	0.12
8	4.02	4.73	5.87	5.02	5.13	3.94	0.31	0.35	0.40	0.14	0.10
9	3.99	4.48	5.35	4.48	4.20	3.07	0.28	0.28	0.34	0.10	0.10
10	3.75	4.31	4.85	3.98	3.43	2.53	0.23	0.23	0.32	0.10	0.10
Total 1st 100 cc.	41.36	52.61	69.71	74.45	88.02	79.92	7.35	8.27	13.48	6.22	2.72*

\* See note to same acid in Table IV.



1,500	95.38	99.55
1,600	96.24	99.69
1,700	96.94	99.78
1,800	97.51	99.85
1,900	97.97	99.90
2,000	98.35	99.93
2,100	98.66	99.95
2,200	98.91	99.97
2,300	99.11	99.98
2,400	99.28	99.99
2,500	99.41	100.00
2,600	99.52	
2,700	99.61	
2,800	99.68	
2,900	99.74	
3,000	99.79	
3,100	99.83	
3,200	99.86	
3,300	99.89	
3,400	99.91	
3,500	99.93	
3,600	99.94	
3,700	99.95	
3,800	99.96	
3,900	99.97	
4,000	99.98	
4,100	99.99	
4,200	100.00	



2,400	99.11	99.99	
2,500	99.27	100.00	
2,600	99.40		
2,700	99.51		
2,800	99.60		
2,900	99.67		
3,000	99.73		
3,100	99.78		
3,200	99.82		
3,300	99.85		
3,400	99.88		
3,500	99.90		
3,600	99.92		
3,700	99.93		
3,800	99.94		
3,900	99.95		
4,000	99.96		
4,100	99.97		
4,200	99.98		

However, to prove this point completely it was deemed necessary to redistil the acids, collect the distillate in successive 10 cc. fractions, and titrate each fraction with 0.1 N KOH. The results obtained are recorded in Table III.

Each of the titrations of the successive 10 cc. fractions was divided by the value obtained from the original titration of the total acid and the result multiplied by 100. The figure so obtained was added to the sum total of the titrations of all preceding 10 cc. fractions treated in the same manner. Table IV shows the results obtained by this process.

Now, conceding that the percentage figure for the first 10 cc. fraction is the distilling constant for each acid (or is that percentage of the residual acid which distills over in each successive 10 cc. fraction), it is possible to carry out some interesting calculations.

#### *Propionic Acid.*

			100.00 - 7.03 = 92.97
$92.97 \times 7.03 = 6.54^*$	$7.03 + 6.54 = 13.57$	$92.97 - 6.54 = 86.43$	
$86.43 \times 7.03 = 6.08$	$13.57 + 6.08 = 19.65$	$86.43 - 6.08 = 80.35$	
etc.			

\* Actually,  $\frac{92.97 \times 7.03}{100} = 6.54$ . The use of the divisor, 100, has been omitted for the sake of brevity.

Carrying out this system of subtracting the distilling constant, as represented by the percentage figure for the first 10 cc. fraction of each acid from 100, multiplying the percentage remaining by the distilling constant and dividing by 100, subtracting the figure so obtained from the percentage left, multiplying, and so on (accompanied by the addition of each quotient to the sum total of those preceding), we should have for each acid a column of figures, as in Table V.

The titrations made on the first 100 cc. fractions of distillate must, of course, be considered as relatively more accurate than those made on the first 10 cc. fractions, especially in the case of the higher and less soluble members of the series. Therefore, Table VI has been inserted. As in Table V, the same principle of calculation was used, but the distilling constants for the 100 cc. fractions (or the percentage amount of each acid distilling over into the first 100 cc. fraction) as given in Table I were employed.

These are simple logarithmic functions and they may be expressed on a simple logarithmic chart, as in Fig. 1.

Since the lines on the chart were drawn in accordance with the data obtained by titration of the first 100 cc. fraction, it will be noted that the line indicating the amount of *n*-caproic acid present in the first 10 cc. fraction of distillate crosses the

# Mixtures of Acids.

Formic

cc Distillate

500

400

300

200

100

90

80

70

60

50

40

30

20

10

n-Butyric

Isobutyric

Isovaleric

n-Caproic

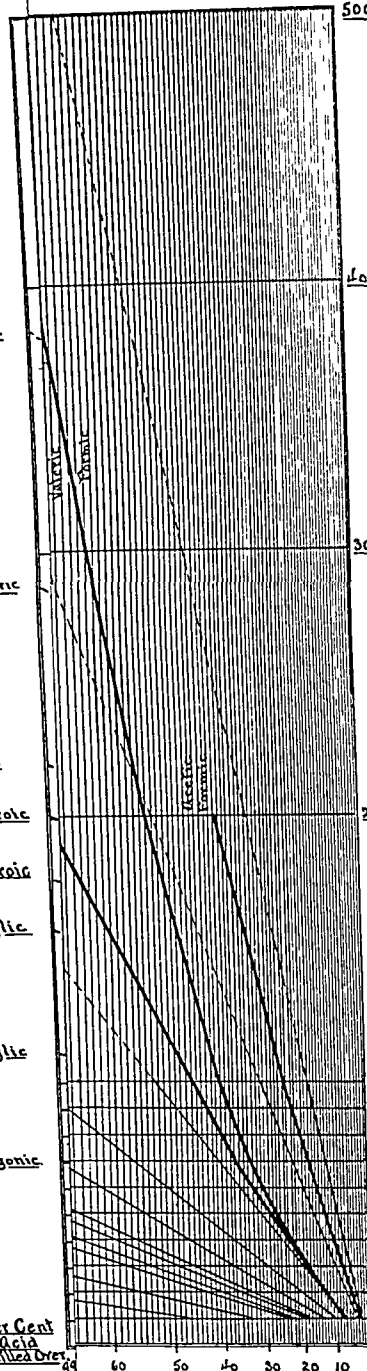
Isocaproic

Heptylic

Caprylic

Palargonic

Per Cent  
Acid  
Distilled Over





However, to prove this point completely it was deemed necessary to redistil the acids, collect the distillate in successive 10 cc. fractions, and titrate each fraction with 0.1 N KOH. The results obtained are recorded in Table III.

Each of the titrations of the successive 10 cc. fractions was divided by the value obtained from the original titration of the total acid and the result multiplied by 100. The figure so obtained was added to the sum total of the titrations of all preceding 10 cc. fractions treated in the same manner. Table IV shows the results obtained by this process.

Now, conceding that the percentage figure for the first 10 cc. fraction is the distilling constant for each acid (or is that percentage of the residual acid which distills over in each successive 10 cc. fraction), it is possible to carry out some interesting calculations.

*Propionic Acid.*

		100.00 - 7.03 = 92.97
$92.97 \times 7.03 = 6.54^*$	$7.03 + 6.54 = 13.57$	92.97 - 6.54 = 86.43
$86.43 \times 7.03 = 6.08$	$13.57 + 6.08 = 19.65$	86.43 - 6.08 = 80.35
etc.		

\* Actually,  $\frac{92.97 \times 7.03}{100} = 6.54$ . The use of the divisor, 100, has been omitted for the sake of brevity.

Carrying out this system of subtracting the distilling constant, as represented by the percentage figure for the first 10 cc. fraction of each acid from 100, multiplying the percentage remaining by the distilling constant and dividing by 100, subtracting the figure so obtained from the percentage left, multiplying, and so on (accompanied by the addition of each quotient to the sum total of those preceding), we should have for each acid a column of figures, as in Table V.

The titrations made on the first 100 cc. fractions of distillate must, of course, be considered as relatively more accurate than those made on the first 10 cc. fractions, especially in the case of the higher and less soluble members of the series. Therefore, Table VI has been inserted. As in Table V, the same principle of calculation was used, but the distilling constants for the 100 cc. fractions (or the percentage amount of each acid distilling over into the first 100 cc. fraction) as given in Table I were employed.

These are simple logarithmic functions and they may be expressed on a simple logarithmic chart, as in Fig. 1.

Since the lines on the chart were drawn in accordance with the data obtained by titration of the first 100 cc. fraction, it will be noted that the line indicating the amount of *n*-caproic acid present in the first 10 cc. fraction of distillate crosses the

# Mixtures of Acids.

Formic

cc Distillate

50

40

30

20

10

5

2

1

0.5

0.2

0.1

0.05

0.02

0.01

n-Butyric

Isobutyric

Valeric

n-Caproic

Isocaproic

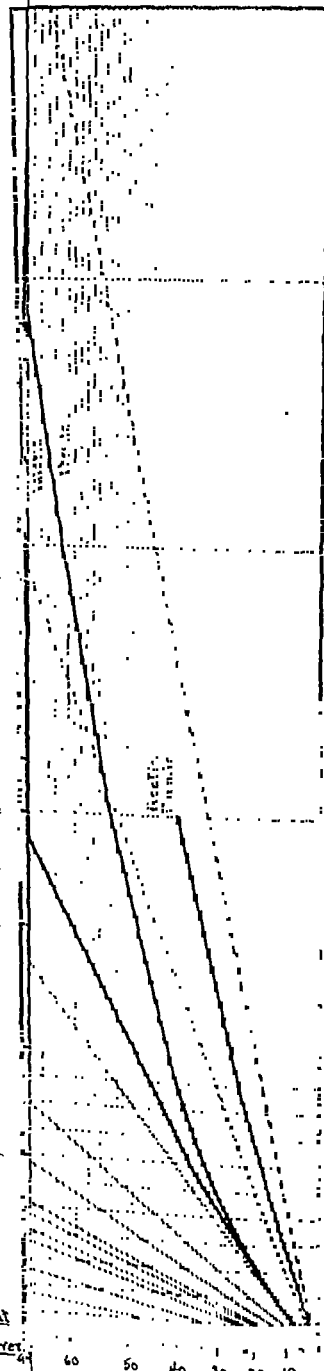
Heptylic

Caprylic

Palargenic

Per Cent  
Acid  
Distilled Over

40 60 50 40 30 20 10





10 cc. point on the chart at a number corresponding approximately to 20.00 per cent, which would calculate out to a distilling constant of 89.26 for the first 100 cc. fraction as against 89.75 determined by actual titration. A distilling constant of 20.00 per cent for the first 10 cc. fraction of distillate for *n*-caproic acid would correspond (in the case of the data in Table III for this acid) to a titration figure of 1.64 cc. of 0.1 N KOH instead of 1.85 cc. as noted in the table. Similarly, the line for isocaproic acid would cross the 10 cc. point at a position corresponding approximately to 23.00 per cent. This would calculate out to a distilling constant of 92.67 for this acid as against 92.60 as determined by actual titration. In Table III, under the caption for this acid, the titration should be 2.05 cc. of 0.1 N KOH instead of 1.92 cc.

The paths of the individual acids have been drawn on the chart as far as the first 100 cc. fraction of distillate, and continued with dotted lines as far as the fifth 100 cc. fraction.

The identification of a single acid by this method of steam distillation requires little or no explanation. It is necessary merely to titrate the total amount of acid present with 0.1 N alkali, noting the number of cc. required, and then arrange the distillation so that the amount of pure acid contained in the total 150 cc. volume in the distilling flask will be about 0.5 cc. For distillation the acid is, of course, liberated with an equivalent amount of 0.1 N or 0.2 N sulfuric acid. Any convenient amount of distillate, say 100 cc., is collected. This is titrated with the 0.1 N alkali and the figure so obtained is divided by that obtained in the first titration representing the total amount of acid distilled. The result will be the distilling constant of the acid for the first 100 cc. fraction of distillate, and a look at the chart will reveal the nature of the acid distilled.

Now it can readily be seen that mixtures of acids upon distillation must give curved instead of straight lines when depicted on the chart. With neighboring acids these curved lines will be very flat while the further removed from each other the acids are in their relative positions on the chart, the greater will be the bulge in the curve. Four examples are given to show the application of this principle to the actual analysis of mixtures of two acids.

## Example 1.

*Mixture of Formic and Acetic Acids.*

0.5 cc. of formic acid required	128.9 cc. of 0.1 N KOH ( $\frac{1}{2}$ = 64.45 cc.)
0.5 " " acetic " "	84.1 " " 0.1 " " ( $\frac{1}{2}$ = 42.05 " )
Total	213.0 " " 0.1 " " ( $\frac{1}{2}$ = 106.50 " )

The potassium salt solution of each acid was made up to a volume of 200 cc. in a graduated flask. A 100 cc. aliquot was taken from each flask, these two aliquots were mixed, and the 200 cc. volume so obtained was evaporated on the water bath to a volume of less than 30 cc. The residue was washed into a 100 cc. graduate, care being taken that the total volume in the graduate did not exceed 40 cc. From a burette was added enough 0.1 N sulfuric acid to bring the volume in the graduate to 100 cc. This was poured into the distilling flask, and then sufficient 0.1 N sulfuric acid was delivered into the graduate to make the total amount of acid used about 108 cc. (a slight excess to make sure that all the fatty acid would be liberated). A little water now added to the graduate brought the volume to 50 cc., and this was added to the 100 cc. already in the distilling flask. The acid so liberated and in solution in a volume of 150 cc. was distilled and the distillate collected in 10 cc. fractions for the first 100 cc. of the distillate, the second 100 cc. fraction of distillate being collected in a 100 cc. graduated flask. The data obtained are shown in Table VII.

TABLE VII.

*Mixture of Formic and Acetic Acids.*

Collected, cc.....	10	10	10	10	10	10	10	10	10	10	100
0.1 N KOH, cc...	2.63	2.71	2.64	2.59	2.53	2.50	2.38	2.30	2.18	2.15	17.50
Acid, * per cent..	2.47	2.54	2.48	2.43	2.38	2.35	2.23	2.16	2.05	2.02	16.43
By addition, collected, cc.....	10	20	30	40	50	60	70	80	90	100	200
Acid, per cent..	2.47	5.01	7.49	9.92	12.30	14.65	16.88	19.04	21.09	23.11	39.54

\* Figures obtained by dividing the number of cc. of 0.1 N KOH required for each 10 cc. fraction by the total titration, i.e., 106.5, and multiplying by 100.

*Problem.*—Given a mixture of two acids one of which, formic, has a distilling constant of 17.89, and another, acetic, which has a distilling constant of 30.75 (in both cases for the first 100 cc. fraction of distillate) in what proportion must they be mixed so that the mixture will have a distilling constant of 23.11 (for the first 100 cc. fraction of the distillate).

As already stated this calculation is one of the easiest, is known in every laboratory, and may be conveniently expressed on paper as follows:

<i>Mixture.</i>	
23.11	
<i>Formic.</i>	<i>Acetic.</i>
17.89	30.75
-5.22	+7.64

Therefore, of 12.86 parts in all (corresponding to 106.5 cc. of 0.1 N KOH), 5.22 parts will represent the acetic acid and 7.64 parts will represent the formic acid.

$$\frac{106.5}{12.86} = 8.2815 \text{ cc. of 0.1 N KOH for each part.}$$

$$8.2815 \times 5.22 = 43.23 \text{ cc. of 0.1 N KOH for the acetic acid.}$$

$$8.2815 \times 7.64 = 63.27 \text{ " " 0.1 " " " " formic "}$$

$$0.0060042 \times 43.23 = 0.2596 \text{ gm. of acetic acid determined.}$$

$$0.0060042 \times 42.05 = 0.2525 \text{ " " " " taken.}$$

$$0.0046021 \times 63.27 = 0.2912 \text{ gm. of formic acid determined.}$$

$$0.0046021 \times 64.45 = 0.2966 \text{ " " " " taken.}$$

### *Example 2.*

#### *Mixture of Formic and Acetic Acids.*

1 cc. of formic acid required 215.11 cc. of 0.1 N KOH.

1 " " acetic " " 171.52 " " 0.1 " "

Total 386.63 " " 0.1 " "

This determination was intentionally made in a very rough manner with samples of acids different from those used in Example 1. 1 cc. of the formic acid was titrated with the 0.1 N KOH and the number of cc. required was noted as above. The titrated portion was then rejected. The same sample of acetic acid was used here as that indicated in Table III. 1 cc. of each of the acids was then added to 150 cc. of water and the distillation was carried out. The conditions of this distillation vary considerably from those in the previous case. Four times the amount of acid was distilled and, using the titration for total acid as indicated above, the acidity of the mixture distilled could be considered as only approximately correct. The distillate was collected as before; i.e., in 10 cc. fractions (Table VIII).

TABLE VIII.  
*Mixture of Formic and Acetic Acids.*

Collected, cc.....	10	10	10	10	10	10	10	10	10	10
0.1 N KOH, cc....	10.33	10.17	10.10	9.80	9.45	9.42	9.13	8.88	8.62	8.32
Acid, per cent....	2.67	2.63	2.61	2.53	2.44	2.44	2.36	2.30	2.23	2.15
By addition, collected, cc.....	10	20	30	40	50	60	70	80	90	100
Acid, per cent....	2.67	5.30	7.91	10.44	12.88	15.32	17.68	19.98	22.21	24.36

Mixture.	
Formic.	Acetic.
17.89	30.75
-6.47	+6.39

Therefore, of 12.86 parts in all (corresponding to 386.63 cc. of 0.1 N KOH), 6.47 parts will represent the acetic acid and 6.39 parts will represent the formic.

$$\frac{386.63}{12.86} = 30.07 \text{ cc. of } 0.1 \text{ N KOH for each part.}$$

$$30.07 \times 6.47 = 194.55 \text{ cc. of } 0.1 \text{ N KOH for the acetic acid.}$$

$$30.07 \times 6.39 = 192.15 \text{ " " } 0.1 \text{ " " " " formic "}$$

$$0.0060042 \times 194.55 = 1.1684 \text{ gm. of acetic acid determined.}$$

$$0.0060042 \times 171.52 = 1.0298 \text{ " " " " taken (approximately).}$$

$$0.0046021 \times 192.15 = 0.8843 \text{ gm. of formic acid determined.}$$

$$0.0046021 \times 215.11 = 0.9900 \text{ " " " " taken (approximately).}$$

#### Example 8.

##### *Mixture of Acetic and n-Butyric Acids.*

0.5 cc. of acetic acid required	84.8	cc. of 0.1 N KOH	( $\frac{1}{2}$ = 42.40 cc.)
0.5 " " n-butyric " "	53.08	" " 0.1 " "	( $\frac{1}{2}$ = 26.54 " )
Total	137.88	" " 0.1 " "	( $\frac{1}{2}$ = 68.94 " )

After evaporating the combined potassium salt solutions of the acids to a volume somewhat less than 100 cc., the residue was made up to a volume of 100 cc. in a graduated flask and a 50 cc. aliquot was taken. This aliquot was made up to a volume of 150 cc., using a graduate, with the

requisite amount of 0.1 N sulfuric acid and water, and then distilled. The 10 cc. fractions collected gave the titrations shown in Table IX.

TABLE IX.  
*Mixture of Acetic and n-Butyric Acids.*

collected, cc.....	10	10	10	10	10	10	10	10	10	10	100	100	100	100
1 N KOH, cc...	4.68	4.30	3.92	3.63	3.31	2.96	2.62	2.28	2.00	1.89	14.80	7.84	4.91	2.97
acid, per cent.	6.79	6.24	5.69	5.27	4.80	4.30	3.80	3.31	2.90	2.74	21.47	11.37	7.12	4.31
by addition, collected, cc.....	10	20	30	40	50	60	70	80	90	100	200	300	400	500
acid, per cent.	6.79	13.03	18.72	23.99	28.79	33.09	36.89	40.20	43.10	45.84	67.31	78.68	85.80	90.11

<i>Mixture.</i>	
45.84	
Acetic.	n-Butyric.
30.75	69.88
-15.09	+24.04

Of 39.13 parts in all (corresponding to 68.94 cc. of 0.1 N KOH), 24.04 parts will represent the acetic acid and 15.09 parts will represent the n-butyric.

$$\frac{68.94}{39.13} = 1.762 \text{ cc. of 0.1 N KOH for each part.}$$

$$1.762 \times 24.04 = 42.36 \text{ cc. of 0.1 N KOH for the acetic acid.}$$

$$1.762 \times 15.09 = 26.59 \text{ " " 0.1 " " " n-butyric acid.}$$

$$0.0060042 \times 42.36 = 0.2543 \text{ gm. of acetic acid determined.}$$

$$0.0060042 \times 42.40 = 0.2546 \text{ " " " " taken.}$$

$$0.0088084 \times 26.59 = 0.2342 \text{ gm. of n-butyric acid determined.}$$

$$0.0088084 \times 26.54 = 0.2338 \text{ " " " " taken.}$$

Example 4.  
*Mixture of Formic and Isovaleric Acids.*

0.3 cc. of formic acid required	74.95 cc. of 0.1 N KOH ( $\frac{1}{2}$ = 37.48 cc.)
0.4 " " isovaleric " "	34.73 " " 0.1 " " ( $\frac{1}{2}$ = 17.37 " )
Total	109.68 " " 0.1 " " ( $\frac{1}{2}$ = 54.85 " )

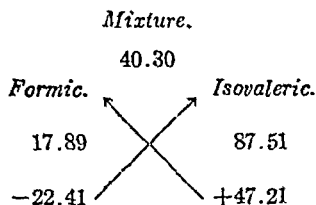


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The potassium salt solutions of both acids were combined, evaporated to a low bulk, made up to 100 cc. in a graduated flask, a 50 cc. aliquot was taken, about 56 cc. of 0.1 N sulfuric acid were added, and enough water to make the total volume 150 cc. The mixture was then distilled. The results obtained are shown in Table X.

TABLE X.  
*Mixture of Formic and Isovaleric Acids.*

Collected, cc.....	10	10	10	10	10	10	10	10	10	10	100	100	100	100
0.1 N KOH, cc.,	4.11	9.36	2.86	2.47	2.10	1.87	1.58	1.39	1.26	1.10	6.98	4.24	3.08	2.36
Acid, per cent.	7.49	6.13	5.22	4.50	3.83	3.41	2.88	2.53	2.30	2.01	12.73	7.73	5.62	4.30
cc. col- d, cc.....	10	20	30	40	50	60	70	80	90	100	200	300	400	500
Acid, per cent.	7.49	13.62	18.84	23.34	27.17	30.58	33.46	35.99	38.29	40.30	53.03	60.76	66.35	70.63



The mixture consists of 47.21 titration parts of formic acid and 22.4 titration parts of isovaleric acid.

$$\frac{54.85}{69.62} = 0.7878 \text{ cc. of } 0.1 \text{ N KOH for each part.}$$

$$0.7878 \times 47.21 = 37.19 \text{ cc. of } 0.1 \text{ N KOH for the formic acid.}$$

$$0.7878 \times 22.41 = 17.65 \text{ " " " " " " isovaleric acid.}$$

$$0.0046021 \times 37.19 = 0.1712 \text{ gm. of formic acid determined.}$$

$$0.0046021 \times 37.48 = 0.1725 \text{ " " " " taken.}$$

$$0.0102105 \times 17.65 = 0.1802 \text{ gm. of isovaleric acid determined.}$$

$$0.0102105 \times 17.37 = 0.1774 \text{ " " " " taken.}$$

The curves illustrating these four examples have been drawn on the logarithmic chart. In the curve drawn for Example 4, for instance, it will be seen that the line crosses out acetic acid as a possible component of the mixture and then reaches a point on the chart where it begins to run parallel with the line which indicates formic acid. This identifies

absolutely the lower acid of the mixture and also gives some indication of the nature of the other acid. It will be seen in the case of Example 4 that the point reached where the line begins to run parallel is about 500 cc., showing that the other acid has run out of the mixture. Turning to Table V and reading across the table from the 500 cc. figure it will be found that the acid due to run out at this point is isovaleric. This is a little uncertain, however, and the qualitative tests given on page 467 are made use of to ascertain the nature of the higher acid of the mixture.

### *The Steam Distillation.*

It might at first appear difficult, if not impossible, to so arrange the steam distillation that the volume in the distilling flask would be maintained at 150 cc. throughout the course of the distillation. In actual practice, however, this proved to be a very simple matter.

For the generation of the necessary steam the regulation steam-can heated by a gas burner was not tried because it seemed probable that such an arrangement would not suffice to maintain a steady and uninterrupted flow of steam to the distilling flask. The apparatus used for the purpose is one suggested by Lassar-Cohn:<sup>4</sup>

"The steam . . . . is generated in a glass flask of 1 or 2 liters' capacity. This is half filled with water and, after the addition of a few drops of sulfuric acid, several pieces of zinc are added. During the boiling there occurs a gentle evolution of hydrogen and a regular and continuous passage of steam is thus obtained unaccompanied with bumping and tossing of the boiling water. If the suggested addition of sulfuric acid and zinc be neglected the water boils with much tossing and a very irregular passage of steam will result."

### *Technique of the Method.*

The current to both hot plates is turned on and also the water leading to the condenser. Flask A is disconnected from the apparatus and into it are slid eight or ten pieces of stick zinc each about 1 inch in length. The flask is filled with ordinary water as hot as it can be procured to about the mark indicated in Fig. 2, and it is then tilted to get a fairly uniform distribution of the pieces of zinc over the bottom of the flask. The flask is

<sup>4</sup> Lassar-Cohn, *Arbeitsmethoden für organisch-chemische Laboratorien*, Hamburg, 1903, 3rd edition, p. 36.

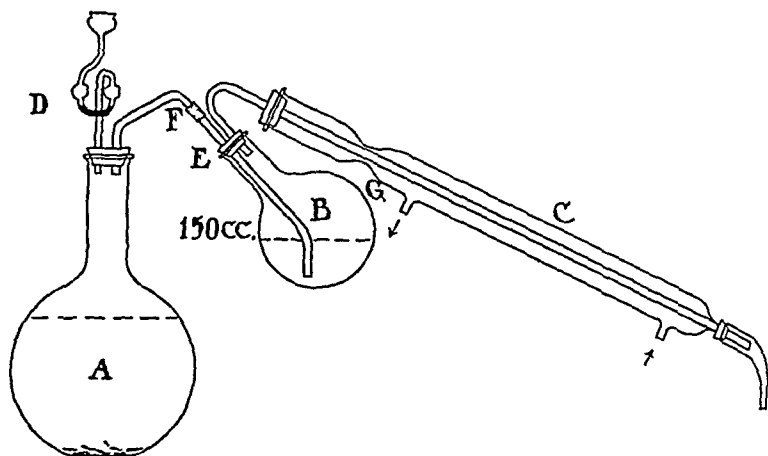


FIG. 2. Apparatus for use in the steam distillation. A, the flat-bottomed flask used for generating the steam, is of 2 liters' capacity. B, the distilling flask in which the volume of liquid is kept constantly at 150 cc. throughout the course of the distillation, is a round-bottomed one of 500 cc. capacity. Ordinary well fitting corks are used in the necks of these two flasks, and to connect the distilling flask with C, an ordinary Liebig condenser. D is a mercury seal made by pouring a little mercury into the thistle tube (there is very little pressure on this seal during the distillation). The glass tubes leading to and from Flask B are of as large a bore as the cork E will conveniently take (the ones used in this laboratory measured  $\frac{1}{8}$  inch, inside diameter). F is a piece of rubber tubing to connect A with B. The tube leading from B reaches to a little below the neck of the condenser (at G).

Flask A is heated by an electric hot plate having a heating surface 6 inches in diameter, and during the distillation this plate is kept with the current at "full." Flask B is heated with a similar hot plate  $4\frac{1}{2}$  inches in diameter. This plate is kept at "full" until the distillation is well started on its way. when it is turned to the next lowest step, "medium." This second hot plate may be placed on an iron ring which is clamped rather loosely to a stand so that it will swivel back out of the way when it is desired to remove Flask B. The apparatus is set up in a corner of the laboratory free from strong draughts and, when the distillation is once started, will run along for hours with little or no change in the 150 cc. volume. When the distillate is collected in 100 cc. fractions the usual work of the laboratory may be carried on without interruption. It is suggested that a trial distillation be made with 0.5 cc. of glacial acetic acid in 150 cc. of distilled water for practice in the use of the method and to determine the correct adjustment of the hot plates.

now placed on the larger hot plate, about 5 cc. of sulfuric acid (1:5) are added, the connecting cork is inserted in the neck of the flask which is then allowed to stand until the water boils vigorously but quietly.

The mixture of the acids in solution is titrated with the 0.1 N alkali solution and a note is made of the number of cc. required. It is advisable to have dissolved in the 150 cc. volume to be distilled an amount of acid equivalent to about 0.5 cc. of the pure acid.<sup>5</sup> A titration figure of 75 cc. of 0.1 N alkali will about represent an average amount in the case of the lower acids of the series, and 60 cc. if there is reason to believe that the mixture consists of the higher ones. It is now possible to figure the amount of dilution or concentration necessary so that the volume of the aliquot taken plus the volume of 0.1 or 0.2 N sulfuric acid required to liberate the fatty acids plus the requisite amount of water will make the total volume 150 cc. Here it may be well to observe that while a slight excess of acid over and above the amount necessary to liberate the fatty acids does no harm, a large excess will increase the rate of distillation of these acids to such a degree that the accuracy of the determination will be appreciably affected. The 150 cc. volume for distillation is made up in an ordinary 100 cc. graduate, in the manner described in the examples of distillations, and then transferred to the distilling flask.

As soon as the steam is seen issuing from the tube leading from the steam generating flask, A, the distilling flask, B, with its contents is attached to the apparatus, the smaller hot plate is swung into its supporting position under the flask, and a mark corresponding to the level of the liquid inside is made on the flask with a blue pencil. The distillation is then carried out. If oil drops or flakes collect in the condenser during the course of the distillation some one or more of the higher acids are present and the supply of cold water flowing through the condenser is turned off at once. The distillate is collected in two 10 cc. graduates using these alternately and reading to the lower meniscus for the 10 cc. mark. The first graduate, when filled to the mark, is removed and the second is substituted. The contents of the

<sup>5</sup> There is, however, considerable latitude in this, especially when the lower and more commonly occurring members of the series, which are more soluble, are distilled. (See the foregoing examples of distillations.)

first graduate are poured into a small Erlenmeyer flask and the graduate is washed out with three or four changes of distilled water. These washings are added to the contents of the flask. The graduate is then flipped vigorously to dislodge the drops of water from it, and set aside in an inverted position until required again. While waiting for the second graduate to fill the first fraction is titrated with the same 0.1 N alkali solution used for the total titration and the figure so obtained is divided by that representing the total titration. The result is multiplied by 100. The figure so obtained is the percentage amount of acid distilled into the first 10 cc. fraction of the distillate—the distilling constant.<sup>6</sup> It has been shown in the foregoing pages that the rate of distillation of a single acid is constant while, in the case of a mixture of acids, the rate of distillation is variable, depending upon the mixture of the acids.

After collecting the tenth 10 cc. fraction a 100 cc. graduated flask is placed under the condenser and the distillate from this point is collected in 100 cc. fractions. These are titrated in the same manner as the foregoing 10 cc. fractions, using phenolphthalein as indicator. The flasks are also washed out with distilled water, as in the case of the 10 cc. graduates, and used alternately. If fat particles adhere to the walls of the flasks they are cleaned out with bichromate mixture, washed thoroughly with distilled water, and then turned upside down to drain until needed for the succeeding fraction.

The number of cc. of 0.1 N alkali required for each succeeding fraction is divided by the total titration and the figure so obtained, after multiplying by 100, is added to the sum total of the figures preceding, which were obtained in the same manner (see examples of distillations). The figure representing the sum obtained with each successive addition is pointed off at its corresponding position on the logarithmic chart until the line of direction so indicated reaches a point from which it runs parallel with that of some one acid. This identifies with certainty the lower acid of the mixture and it is, of course, unnecessary to carry the distillation further. The point at which the line begins its parallel course is the exact place at which the other, and higher, acid of

<sup>6</sup> If the distillate is being collected in 100 cc. fractions this figure will be, of course, the distilling constant of the first 100 cc. fraction.

the mixture has run out. A reference to Table V will give an approximate idea as to which one it is. To establish definitely the nature of this acid, however, the included colorimetric tests must be used. A reference to the examples of distillations given in this paper will indicate the nature of the calculation necessary for determining the amounts of acids comprising the mixture.

The application of this method of steam distillation for the identification and determination of acids in solutions which cannot be titrated directly on account of their dark color, or for some other reason, is obvious. The solution is distilled directly from a total volume of 150 cc. and the distillates are collected either in 10 cc. or 100 cc. fractions. A calculation based on the following will illustrate the procedure.

*Formula for Determination of the Total Amount of Acid Originally Present from the Titrations of the First and Second 100 Cc. Fractions of Distillate.*

$t$  = the total titration to be calculated (unknown).  
 $t_1$  = the titration of the first 100 cc. fraction (known).  
 $t_2$  = " " " " second 100 cc. " "  
 $c$  = the distilling constant (unknown).

then:

$$c = \frac{t_1}{t}, c = \frac{t_2}{t - t_1}, \text{ or } \frac{t_1}{t} = \frac{t_2}{t - t_1}, t = \frac{t_1^2}{t_1 - t_2}$$

As an example (an unknown amount of acetic acid really requires 171.52 cc. of 0.1 N alkali):

First 100 cc. fraction of distillate ( $t_1$ ) required 52.74 cc. of 0.1 N KOH.

Second 100 " " " " ( $t_2$ ) " 36.52 " " " "

Difference 16.22

$t = \frac{(52.74)^2}{16.22} = 171.49$  cc. of 0.1 N KOH (representing the acidity of the acid solution distilled).

$$c = \frac{t_1}{t} \text{ or } \frac{52.74}{171.49} = 30.75 \text{ (distilling constant).}$$

Conclusion: The acid originally present was acetic, and  $0.0060042 \times 171.49 = 1.0297$  gm. of this acid present in the liquid distilled.

Confirmation: By the qualitative colorimetric method.

### *Colorimetric Tests.*

These tests are based upon results obtained while working with a colorimetric test for the identification of the fatty acids

proposed by Agulhon.<sup>7</sup> The principle involved in this test of Agulhon's is good, but the method of carrying it out proved unsatisfactory.

A special study was made of the solubility of various metallic salts of the volatile fatty acids in various organic solvents. For this purpose the copper, iron, nickel, cobalt, chromium, manganese, and uranium salts of these acids were used. It was found that only the salts of iron and copper respond satisfactorily to tests of this nature. As a result of this investigation Agulhon's test has been rearranged, and the range has been somewhat extended through the addition of one or two other solvents. Agulhon states that his test may be nullified to some extent by the formation of insoluble copper salts with the use of copper sulfate, the reagent he indicates. This difficulty has been entirely removed by the substitution of copper chloride.

There is needed for this test a solution of iron chloride not weaker in strength than 2 per cent nor stronger than 3 per cent, and a solution of copper chloride of a strength approximating 0.1 N.

The 10 cc. fractions collected in the distillations are not rejected after the titration with the 0.1 N alkali but are made up to a certain volume so that the strength of the solution is approximately 1.5 per cent of the alkali salt of the fatty acid.<sup>8</sup> This volume may be obtained either by dilution, if too strong, or by evaporating off the excess of water, if too weak.

The test is made in the following manner. 2 cc. of the 1.5 per cent solution of the alkali salts of the fatty acids are trans-

<sup>7</sup> Agulhon, H., *Solubilité de certains sels métalliques des acides gras volatils dans les solvants organiques,—Application à la détermination qualitative de ces acides*, *Bull. Soc. chim.*, 1913, series 4, xiii, 404.

<sup>8</sup> Some judgment of the chemist is here called for. The first fractions of the distillate should be tested for the higher acids of the series and the last fractions for the lower acids. Unless there is some idea as to the nature of the acid, or acids, present in these fractions so that the corresponding factors may be used to obtain the approximate strength solution of the alkali salts of the fatty acids, the factor for propionic acid, which will about strike an average, may be used to multiply the number of cc. of 0.1 N alkali required to neutralize the fraction. When once prepared the alkali salt solutions of the volatile fatty acids should be tested within a reasonably short time since it has been found that molds may begin to grow in them after a few days.

ferred by means of a pipette into a small test-tube (tubes 5 inches long and  $\frac{1}{2}$  inch in diameter were used in this laboratory) and from 0.5 to 1 cc. of the special solvent added, forming a supernatant layer. This mixture is then treated, drop by drop, with agitation, with either the iron chloride or copper chloride test solution, as indicated. The tube then remains at rest for a few moments and the result is noted.

### *Orientation Test.*

To 2 cc. of the 1.5 per cent solution of the potassium or sodium salts of the fatty acids under investigation add about 0.5 or 1 cc. of amyl alcohol, stopper with the thumb, agitate the tube vigorously to mix the solvent and the solution, and then hold it under the hot water tap or plunge the tube into hot water, where it is held for several seconds with shaking, until the vapors of amyl alcohol have almost or entirely displaced the column of air from the tube. Cool, and then add one drop of the iron chloride solution. Stopper the mouth of the tube with the thumb, agitate vigorously, and set aside for a minute or two. If a brownish yellow precipitate is formed which hangs suspended in the aqueous liquid even for some minutes on standing, while the layer of amyl alcohol on the surface is colored a light yellow, the acid present may be either *heptylic*, *caprylic*, *pelargonic*, or *capric*. If the precipitate formed shows an inclination to dissolve readily in the solvent, add another drop of the iron chloride solution, stopper with the thumb, agitate vigorously, and again note the result. The iron salt of *propionic* acid is only partly soluble in the solvent. In this case the test will be indicated by a pronounced brownish yellow tinge of the amyl alcohol, by the collection of brown insoluble salts at the junction of the two liquids, while the aqueous solution will be colorless. With *acetic* acid the aqueous solution will be colored a clear urine shade of yellow containing the faintest tinge of red due to the dissolved iron salt of the acid itself, there will be no precipitate on standing, and the amyl alcohol layer will be colorless. With *formic* acid the amyl alcohol layer will be likewise colorless, but the aqueous solution will be cloudy and will give a red precipitate on standing for several minutes. If the amyl alcohol layer has begun to take



on a rich deep red after the addition of successive drops of the iron chloride solution, continue the addition of the drops with agitation until a full intense red has been developed. The iron salts of the three acids, *butyric*, *valeric*, and *caproic* are entirely soluble in the amyl alcohol and give the intense red color.

### *Individual Tests.*

*Formic and Acetic Acids.*—No individual colorimetric test for formic acid could be found. It is necessary to make use of its reducing properties to identify it (the formation of a blue color with nitric acid and bichromate of potash mixture).<sup>9</sup> However, this acid may be identified with certainty in the manner noted under "Orientation Test," and in the steam distillation. Of all the solvents turpentine is the only one found which is able to dissolve the copper salts of formic and acetic acids and bring them to the surface of the aqueous solution.

In the absence of formic and the other acids, acetic acid may easily be identified by the following test. 2 cc. of the alkali salt solution of the fatty acid are placed in a small test-tube and covered with a 0.5 or 1 cc. layer of ordinary turpentine.<sup>10</sup> The approximately 0.1 N copper chloride solution is then added drop by drop from a burette, the mouth of the tube is closed with the thumb, and it is agitated after each addition. The copper salt of acetic acid will be dissolved by the turpentine and brought

<sup>9</sup> Agulhon, Recherche colorimétrique de l'alcool en présence de l'acétone. Réactions colorées de certains groupements organiques en présence d'acides minéraux et de bichromate de potassium, *Bull. Soc. chim.*, 1911, series 4, ix, 881.

<sup>10</sup> The solvent properties of the turpentine may be considerably increased by dissolving in it all the crude resin (rosin, colophonium) it will take up, which is very little. This may be done by triturating the turpentine with the powdered resin in an evaporating dish and decanting off the liquid. Redistilled turpentine cannot be used for this test. This turpentine test may also be used to distinguish between a volatile fatty acid and lactic acid; copper lactate is insoluble in the turpentine which rises as a colorless layer to the surface of the aqueous solution after agitation. It may also be noted here that lactic acid is practically non-volatile by the method of steam distillation described in this paper. The first 100 cc. fraction from 2 gm. of lactic acid dissolved in 150 cc. of water and steam-distilled required but 0.25 cc. of 0.1 N KOH.

to the surface of the solution as a deep greenish blue layer, provided the agitation has not been so great as to form an emulsion.

*Propionic Acid.*—Use the test given under "Orientation Test." The test with amyl alcohol and iron chloride was the only satisfactory one found. At first, add but one drop of the iron chloride solution. This will usually suffice to develop the brownish yellow tinge of the amyl alcohol. At the most, add but a few additional drops. If more than this is added the tendency of the insoluble salts formed is to drag the brown color out of the amyl alcohol, leaving it colorless.

*Butyric and Valeric Acids.*—Make the test as already indicated but use ether as the solvent<sup>11</sup> and iron chloride as the precipitant. If the acid present is butyric the two liquids will emulsify somewhat upon agitation, while the ether layer which eventually rises will be colorless. If the acid is valeric the ether layer will be reddish yellow in color while the aqueous solution will be decolorized. In both cases there will be a collection of insoluble salts at the junction of the two liquids.

*Valeric, Caproic, and Heptylic Acids.*—The first two acids mentioned are the only acids of the series which give color reactions with iron chloride and ether. Make the test as above described and set aside for a minute or two. The iron salts of valeric and caproic acids will color the ether layer a reddish yellow. In the case of the valerate the aqueous solution will be decolorized but very cloudy with insoluble iron salts if the acid present is caproic. In both cases there will be a collection of insoluble salts at the junction of the two liquids. The iron salt of heptylic acid is entirely insoluble in both the ether layer and the aqueous solution and there is no collection of insoluble salts at the junction of the two liquids.

*Valeric and Caproic Acids.*—Perform the test as usual, but with copper chloride and either petrol-ether (40–60°), gasoline, or kerosene, as the solvent. The copper salt of valeric acid is nearly all soluble in the aqueous solution, while the solvent layer rises colorless to the top. With caproic acid the solvent takes on a decided blue color.

*Caproic and Caprylic Acids.*—Perform the test as above de-

<sup>11</sup> It is preferable to use "ether over sodium" for these tests.

scribed, using the solution of iron chloride as precipitant and ethyl acetate free from alcohol as the solvent. If the acid present is caproic the aqueous solution will be decolorized while the solvent will possess a rich iron-red color. The iron salt of caprylic acid is almost entirely insoluble in both the solvent and the aqueous solution. At most, the solvent will possess but a faint yellow color while the aqueous solution will be very cloudy with insoluble salts.

#### SUMMARY.

A new and simple method of steam distillation for the determination of the volatile fatty acids has been devised. This method depends upon the maintenance of the aqueous solution distilled and which contains the acid, or acids, at a constant volume of 150 cc. throughout the course of the distillation. A simple and convenient apparatus for maintaining this volume is described.

By this method of steam distillation the distilling constants of the individual volatile fatty acids are indicated by straight lines when depicted on a simple logarithmic chart. On the other hand, the distilling variables of mixtures of these acids are indicated on the same chart by curved lines. With neighboring acids these curves will be very flat, while the further the acids comprising the mixture are removed from each other in their relative positions in the series the greater will be the bulge in the curve.

The lowest acid of the series in the mixture will be identified with certainty since it will eventually reach a point where it will begin to parallel some straight line on the chart which represents that acid. This same point indicates where the other acid of the mixture has run out and reference to a table given will indicate with a fair degree of probability the nature of this accompanying acid.

The calculations given which are necessary for the determination of the amounts of acid comprising the mixture are of the simplest, thus avoiding all errors. The application of these calculations to the determination of acids present in dark colored acid solutions and in bacteriological media where the acid present cannot be titrated directly has been shown.

A qualitative colorimetric test for the identification of these acids has also been included, and this may be used alone or in conjunction with the steam distillation method.



# THE MECHANISM OF CYTOLYSIS IN SEA URCHIN EGGS.

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Because of its relation to the theory of hemolysis on the one hand and to that of membrane formation in artificial parthenogenesis on the other, the mechanism of cytolysis is a matter of interest and importance to the general physiologist. The purpose of the investigations described below is to determine the character of the change which brings about cytolysis.

In attempting to account for hemolysis, Koeppe<sup>1</sup> advanced the view that the outflow of hemoglobin from the blood cell is the result of the destruction of the limiting membrane of the corpuscle, which he believed to be a lipid substance. Consequently he interpreted hot water hemolysis as a melting of the lipid sac of each corpuscle, and referred fat solvent action to the solution of the lipid shell.

Loeb,<sup>2</sup> considering the question with reference to the sea urchin egg, formulated a theory of cytolysis and membrane formation (superficial cytolysis) which was an elaboration and extension of Koeppe's hypothesis. According to Loeb's view, the lipoids of the cytoplasm are liquefied by various cytolytic agents, and as a result water is absorbed by the cytoplasm. This leads first to membrane formation and, if continued, to complete cytolysis; viz., swelling and clearing of the entire egg.

Von Knaffl,<sup>3</sup> working under Loeb's direction, showed that, as a result of cytolysis, substances giving tests for lecithin flowed

<sup>1</sup> Koeppe, H., *Arch. ges. Physiol.*, 1903, xcix, 50.

<sup>2</sup> Loeb, J. Über den chemischen Charakter des Befruchtungsvorgangs, in Roux, W., *Vorträge u. Aufsätze über Entwicklungsmechn. Organ.*, Leipsic, 1908, pt. ii.

<sup>3</sup> Von Knaffl-Lenz, E., *Arch. ges. Physiol.*, 1908, cxiii, 279.

out of the sea urchin eggs. His results on hot water cytolysis seemed to indicate a melting point, 39–42°C., and thus gave support to a simple liquefaction theory of cytolysis.

As a result of later work,<sup>4</sup> it became evident that there is not a melting point for sea urchin egg lipoids, but rather a liquefaction range, 34–42°C., and further that there is a definite temperature coefficient for the process; *viz.*, 3 for 2°C. or about 200 for an interval of 10°C. This corresponds to a value of about 100,000 for  $\mu$  in Arrhenius' equation:

$$K_1 = K_0 e^{\frac{\mu}{2} \left( \frac{T_1 - T_0}{T_1 T_0} \right)}$$

where  $K_0$  and  $K_1$  are the reaction velocity constants at the absolute temperatures  $T_0$  and  $T_1$ , respectively;  $e$  is the base of the natural logarithms;  $\mu$  is a constant. The above value for  $\mu$  indicates that hot water cytolysis belongs to the same type of reaction as the destruction of bacteria at high temperatures and hot water hemolysis.<sup>5</sup>

While von Knaffl's evidence would indicate that cytolysis is fundamentally a physical process, *i.e.*, a melting or a solution, the magnitude of the temperature coefficient may mean that the process is a chemical one. That cytolysis may be due to increased fluidity of certain egg constituents and at the same time follow the monomolecular law is shown by the fact that Schroeder<sup>6</sup> found that heating a gelatin solution at 100°C. for different lengths of time, reduced the viscosity of the solution at a rate characteristic of a monomolecular reaction.<sup>7</sup> He interpreted the change as due to a hydrolysis of the gelatin and called the phenomenon a "saponification reaction." Furthermore, Dunstan and Mussell<sup>8</sup> in 1911 showed that the courses of a number of reactions, such as acetic anhydride +  $H_2O \rightarrow$  acetic acid, benzoyl

<sup>4</sup> Moore, A. R., *Quart. J. Exp. Physiol.*, 1910, iii, 257.

<sup>5</sup> Arrhenius, S., *Quantitative Laws in Biological Chemistry*, London, 1915, 55.

<sup>6</sup> Schroeder, P. v., *Z. physik. Chem.*, 1903, xlv, 75.

<sup>7</sup> R. S. Lillie uses Schroeder's data on gelation to support the contention that the increase of fluidity in cytolysis is due to physical change. *Biol. Bull.*, 1915, xxviii, 295

<sup>8</sup> Dunstan, A. E., and Mussell, A. G., *J. Chem. Soc.*, 1911, xcix, 565.

chloride +  $\text{H}_2\text{O}$   $\rightarrow$  benzoic acid, acetoacetic ester  $\rightarrow$  equilibrium mixture, aniline + ammonium thiocyanate  $\rightarrow$  phenyl thiocarbamide, were accompanied *pari passu* by regular and progressive changes in viscosity. They were able in these cases to determine the reaction velocity constant for each reaction by means of viscosity measurements, which showed the reactions to be of the first order.

We may regard it as a fact established, that cytolysis is due in the main to an increase in fluidity (decrease in viscosity) of certain cell constituents, probably lipoids. A measure of cytolysis therefore becomes a measure of the fluidity of the system. This increase in fluidity may be due either to a simple melting or to a chemical reaction. From the foregoing it is apparent that our task is to decide between these two possibilities. Stated in another way, our problem becomes: Is cytolysis a monomolecular reaction?

It has been shown<sup>4</sup> that the progress of the cytolysis reaction, *i.e.*, the increase in the percentage cytolized during a given time interval can be followed step by step, simply by removing a pipetteful of eggs from the hot sea water from time to time, putting them into a watch-glass of cold sea water, and afterwards determining the percentage cytolized by counting several hundred eggs in each watch-glass. Such measurements give a basis for deciding the character of the reaction, whether it is a physical process involving only the relation of molecules to each other, or to a chemical reaction involving a transformation within the molecule, such as a hydrolysis.

The fact that the percentage of eggs cytolized at a given temperature,  $T^\circ$ , is a function of the time for which  $T^\circ$  is maintained may be interpreted in one of two ways:<sup>9</sup>

1. The time at  $T^\circ$  necessary for an egg to cytolize may be due chiefly to the age of the egg, in which case the reaction should be slow at first, most rapid when the process is half completed, and fall off to zero at the end. If such were the case, the time plotted as abscissæ against  $\log_e N$  ( $N$ , number of eggs remaining uncytolized at the end of the time interval taken) as ordinates, would give a double curve graph.

<sup>9</sup> Arrhenius, Quantitative Laws in Biological Chemistry, London, 1915, 78.



2. The increase in the number cytolized with time may amount to a certain percentage of the eggs remaining uninjured at the end of each time interval, and therefore be independent of the age or natural resistance of the egg. Such a dependence on the law of probability is shown by chemical reactions. In this case the graph resulting from plotting time as abscissæ against  $\log_e N$  as ordinates must be a straight line.

Figs. 1, 2, 3, and 4 show that hot water cytolysis fulfills the requirements of the second case and is therefore of the nature of a chemical reaction.

The temperature coefficient of the process and the logarithmic relation of the percentage change to time both indicate hot water cytolysis to be of the nature of a chemical reaction. If this conclusion is correct, then it should be possible to determine the order of the reaction.

Using the method described, a large number of measurements of the percentages of cytolysis at various temperatures were made. It is obvious that on account of the variations between the lots of eggs from different individuals, and because we are forced to determine each percentage from a few hundred eggs, the experimental errors are large. Nevertheless, repeated tests made in different seasons, with the fresh unfertilized eggs of the sea urchin, *Strongylocentrotus purpuratus*, have given concordant results. In the table, each one of Experiments 1, 2, 3, and 4 represents results derived from the eggs of one individual sea urchin. Experiments 5 and 6 show the rates of cytolysis in two lots of eggs from the same female, tested at the same time; in Experiment 5 the eggs were normal unfertilized; in Experiment 6 the eggs were kept for 10 minutes in a solution of  $\text{SrCl}_2$   $3/8$  M before being put into the heated sea water.

In testing the hypothesis set forth above, whether cytolysis is due to a monomolecular reaction, the form of the equation used is

$$K = \frac{\log_e N_0 - \log_e N_1}{t_1 - t_0}$$

in which  $N_0$  is the percentage of eggs uncytolized at the beginning of the experiment  $t_0$ ;  $N_1$  is the percentage of eggs uncytolized at the end of a given time  $t_1$ ;  $K$  is the reaction velocity constant. In case the calculations were made from  $t_0 = 0$  the first constants

TABLE I.

$t$	$N$	$\log_e N$	$K = \frac{\log_e N_0 - \log_e N_1}{t_1 - t_0}$
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Experiment 1.  $T = 35^\circ$ .  $t_0 = 10$ .

10	98	4.58	
20	88	4.47	0.011
30	55	4.00	0.029
40	51	3.93	0.022
50	33	3.49	0.027

Experiment 2.  $T = 36^\circ$ .  $t_0 = 10$ .

10	97	4.57	
20	68	4.21	0.036
30	55	4.00	0.0285
40	40	3.68	0.030
60	33	3.49	0.022

Experiment 3.  $T = 39^\circ$ .  $t_0 = 2$ .

2	91	4.51	
4	44	3.78	0.36
6	33	3.49	0.25
8	12.5	2.52	0.33
10	10	2.30	0.28

Experiment 4.  $T = 40^\circ$ .  $t_0 = 4$ .

4	97	4.57	
5	90	4.49	0.080
7	75	4.31	0.086
9	65	4.17	0.080
11	60	4.09	0.069
13	50	3.91	0.073

Experiment 5.  $T = 37.5^\circ$ .  $t_0 = 3$ .

3	100	4.600	
5	99	4.589	0.005
10	95	4.549	0.007
20	85	4.439	0.0095

Average..... 0.0072

Experiment 6.  $T = 37.5^\circ$ .  $t_0 = 0$ .

3	85	4.44	0.053
5	65	4.17	0.086
10	35	3.55	0.105
20	20	2.99	0.080

Average..... 0.081

were invariably low in value. This may be due, as Arrhenius suggests,<sup>10</sup> to the necessity for an incubation period in the reaction.<sup>11</sup> The constants calculated from  $t_0 = t$  of the first experimental reading show a fair degree of agreement. Hence we must conclude that the increase in fluidity which gives rise to cytolysis is due to a simple monomolecular reaction, possibly a hydrolysis. It will be seen that such a conclusion is in accord with Schroeder's theory and with the results of Dunstan's and Mussell's elaborate investigations.

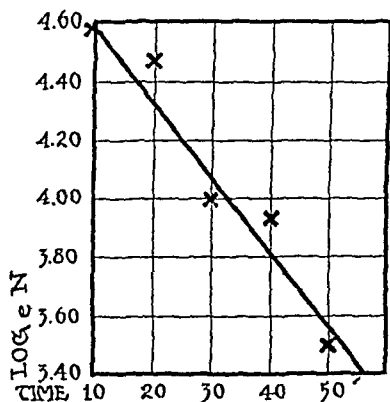


FIG. 1. From Experiment 1.

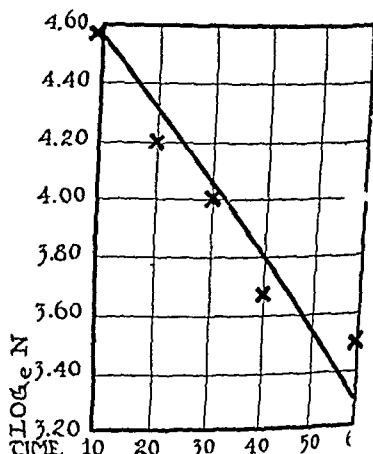


FIG. 2. From Experiment 2.

### *The Effect of Strontium Ions on the Reaction Velocity of Cytolysis*

Loeb<sup>12</sup> has shown that treatment of the unfertilized eggs of the sea urchin with  $\text{SrCl}_2$  solution causes them to form fertilization membranes (superficial cytolysis) when treated with foreign serum. Robertson<sup>13</sup> has shown that  $\text{CaCl}_2$  solution acts in the

<sup>10</sup> Arrhenius, *Quantitative Laws in Biological Chemistry*, London, 1915, 64.

<sup>11</sup> Charging the cylinder of heated sea water with the pipetteful of eggs necessarily reduces the temperature at the outset of the experiment by  $0.2-0.4^\circ\text{C}$ .; experimental temperature is recovered in 3 to 5 minutes.

<sup>12</sup> Loeb, *Artificial Parthenogenesis and Fertilization*, Chicago, 1913, 195.

<sup>13</sup> Robertson, T. B., *Arch. Entwicklungsmechn. Organ.*, 1912-13, xxxv, 88.

same way. The writer found<sup>14</sup> that the eggs of *Strongylocentrotus purpuratus* could be fertilized with starfish sperm if first sensitized with  $\text{SrCl}_2$ , and that the rate of hot water cytolysis increases enormously if the eggs have been kept for a few minutes in  $3/8 \text{ M}$   $\text{SrCl}_2$  solution before being put into the heated sea water. A consideration of Experiments 5 and 6 shows that the magnitude

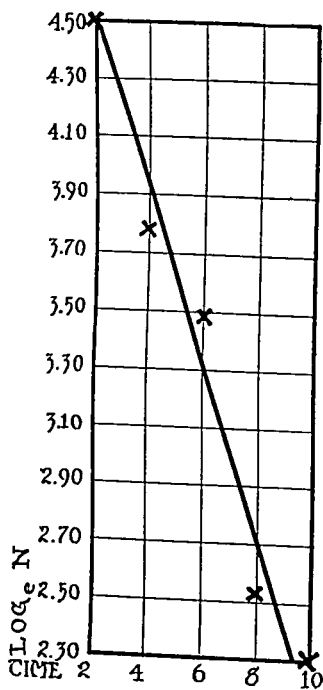


FIG. 3. From Experiment 3.

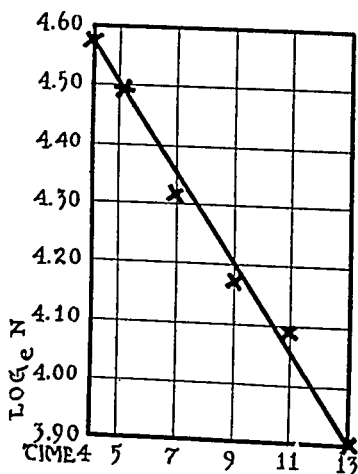


FIG. 4. From Experiment 4.

of the velocity constant of cytolysis at  $37.5^\circ\text{C}$ . for the eggs treated with  $\text{SrCl}_2$  solution is eleven times that of the control. Since the  $\text{SrCl}_2$  adds no energy to the system, it must therefore influence the reaction as a catalyzer. It is interesting and doubtless pertinent to note that Osterhout<sup>15</sup> regards the specific effect of the Ca ion on protoplasm as due to its action as a catalyzer of certain

<sup>14</sup> Moore, *Arch. Entwicklungsmechn. Organ.*, 1913, xxxvii, 433.

<sup>15</sup> Osterhout, W. J. V., *Marine Biological Laboratory Lectures*, Woods Hole, 1916.

metabolic reactions. The suggestion may then be made that the sensitizing action of Sr and Ca ions on sea urchin eggs may be due to the fact that these ions act as catalyzers of the cytolysis reaction.

#### CONCLUSIONS.

1. Hot water cytolysis of the fresh unfertilized eggs of the sea urchin depends upon an increase in the fluidity of certain of the eggs' constituents, which change is caused by a process proceeding at the rate of a reaction of the first order.

2. Strontium ions act as a positive catalyst of the cytolysis reaction. The action of strontium ions in sensitizing sea urchin eggs to foreign sera and to starfish sperm may be due to catalysis of the cytolysis reaction.

# IS LYSINE THE LIMITING AMINO-ACID IN THE PROTEINS OF WHEAT, MAIZE, OR OATS?\*

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(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.)

## PLATE 4.

(Received for publication, November 27, 1916.)

Data published in 1914 by Osborne and Mendel<sup>1</sup> were interpreted by them as indicating that maintenance but not growth of an animal is possible without the amino-acid lysine. This created the impression in the minds of several investigators that this amino-acid is the limiting factor in determining the value of many proteins of vegetable origin.<sup>2</sup> The analytical data secured by Osborne, Van Slyke, Leavenworth, and Vinograd<sup>3</sup> on a number of plant proteins support this interpretation. They conclude that the lysine content of the proteins of the endosperm of wheat, maize, and rice is respectively 1.58, 0.97, and 4.26 per cent of the total nitrogen, and state that "It is rather striking that the figures for the utilizability of these proteins correspond so closely with the lysine which they yield."

Buckner, Nollau, and Kastle<sup>2</sup> interpreted the experimental data of Osborne and Mendel<sup>1</sup> as showing that "lysine is primarily responsible for the stimulation of growth." They fed to chicks such complex mixtures as wheat, wheat bran, sunflower seed, hemp seed, and skim milk, and contrasted the results with those obtained with a ration which consisted of a mixture of barley, rice, hominy, oats, and gluten flour. They interpreted

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325, 334.

<sup>2</sup> Buckner, G. D., Nollau, E. H., and Kastle, J. H., *Am. J. Physiol.*, 1916, xxxix, 162 (and 21); *Kentucky Agric. Exp. Station, Bull.* 197, 1916.

<sup>3</sup> Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

the results of these feeding experiments as making it evident that the marked differences shown by the two lots of chicks in rate of growth and development are to be ascribed to the "differences in the amino-acid content of the two rations and in all probability to differences in the lysine content."

The findings of Osborne<sup>3</sup> that gliadin purified as far as possible still yields 1.21 per cent of lysine naturally invalidate the earlier deductions of Osborne and Mendel that lysine is dispensable from the diet during maintenance.

In more recent work by Osborne and Mendel<sup>4</sup> they employed corn gluten, containing about 1 per cent of lysine, together with 1.33 per cent of  $N \times 6.25$  as "protein-free milk" as the sole source of protein. They compared the growth obtained with this protein mixture with that obtained with rations similar in their make-up except that the corn gluten and "protein-free milk" nitrogen was supplemented with lactalbumin and cottonseed flour respectively. The latter additions are known to yield the amino-acid lysine in relatively high amounts. The authors state that their results confirm the conclusions drawn by Buckner, Nollau, and Kastle respecting the effect of foods high and low in lysine on the growth of chicks.

In the course of our studies directed toward ascertaining the supplementary relationships among the naturally occurring food-stuffs, we have secured data which bear on this subject in a definite way. The results leave no room for doubt that a generalization cannot be safely made that any one amino-acid, as lysine, is present in the protein mixture in our more common vegetable foods in amount relatively smaller than are other essential amino-acids. Indeed we are forced to the conclusion that in the protein mixture of the maize kernel and the oat kernel, lysine certainly is not the essential protein cleavage product which is present in amount so small that it is the limiting factor which determines the biological value of the proteins of these seeds.

Our rations were made up after the following plan. A food mixture consisted of a single grain so supplemented with inorganic salts and butter fat as to be properly constituted for promoting growth and prolonged well-being, except that the protein was too low in amount to promote growth at the normal rate. In

<sup>4</sup> Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 293, 299.

all cases our rations were shown to be made highly efficient for growth by the addition of purified protein alone, when casein was employed. Casein is well known to be a complete protein, and to suffice as the sole source of nitrogen throughout the life of the rat.<sup>5</sup>

Rations made up in this way were fed without the addition of supplementary protein and also with the addition of the incomplete proteins zein and gelatin respectively. The former yields no lysine while the latter yields about 6 per cent.<sup>3</sup> Obviously if lysine were the one amino-acid whose addition is necessary in order to improve the protein mixture of the grain employed in the food mixture there should be a response with growth when gelatin was added and no improvement when zein was added.

For completeness we also present curves of growth showing the effect of adding wheat gluten, the proteins of which yield but little lysine (1.58 per cent)<sup>3</sup> to rations which aside from the wheat gluten addition, derived their protein solely from the wheat, maize, and oat kernels respectively.

Our results may be briefly summarized as follows:

1. Zein does not supplement the protein mixture in the wheat kernel or the maize kernel so as to improve the rate of growth of young rats (Charts 3 and 10).

2. Zein does supplement the proteins of the oat kernel in a surprisingly efficient manner (Chart 7) although it lacks tryptophane and lysine and is one of the poorest of the proteins in cystine. In the proteins of the oat kernel, therefore, the above amino-acids are eliminated as being possibly the essential protein cleavage products present in minimal amount, and determining the plane of intake essential for growth.

<sup>5</sup> This statement rests on our experience reported elsewhere (*J. Biol. Chem.*, 1915, xxiii, 231) of observing growth at about the normal rate on a diet in which 99 per cent of the nitrogen was furnished by casein and 1 per cent by the alcoholic extract of wheat germ, added for the purpose of supplying the still unidentified dietary factor, the water-soluble B. 28 per cent of "protein-free milk," containing 0.76 per cent of nitrogen, which serves the same purpose as our extract, furnished, when fed with 18 per cent of purified proteins, 6.9 per cent of the total nitrogen of the food mixture in forms not further characterized except that we have found it not to be without biological value as a source of the cleavage products of protein (*J. Biol. Chem.*, 1915, xx, 415).



3. Gelatin chemically supplements the protein mixture of both the wheat kernel and oat kernel respectively. Since gelatin contains no tyrosine or tryptophane, and but a trace of cystine, but contains 6 per cent of lysine, it is evident that neither tyrosine, tryptophane, nor cystine is the limiting amino-acid in these grains. It tends to support the view, but does not prove that lysine is the amino-acid whose addition alone to the wheat protein mixture raises the biological value of the latter.

4. The addition of wheat gluten to either the wheat or maize kernel proteins supplements them so as to improve growth. The proteins of the wheat gluten are probably as a mixture qualitatively adequate as respects all of the indispensable amino-acids, although we cannot look upon this point as definitely established. One of its constituents, glutenine, has been shown by Osborne and Mendel to be capable of supporting growth when it furnished 93.2 per cent and "protein-free milk" 6.8 per cent.

It seems probable, therefore, that our combinations of wheat kernel proteins with wheat gluten, and of maize kernel proteins with wheat gluten led to growth because of a higher intake of protein rather than to a supplementary relationship between the proteins from the two sources in the sense of one making good the amino-acid deficiencies of the other. The results of feeding maize proteins with wheat gluten are of particular interest, however, because of their pronounced effect in promoting growth despite the relatively low lysine content of both the wheat and maize proteins.

Gelatin with its high lysine content does not improve the proteins of the maize kernel.

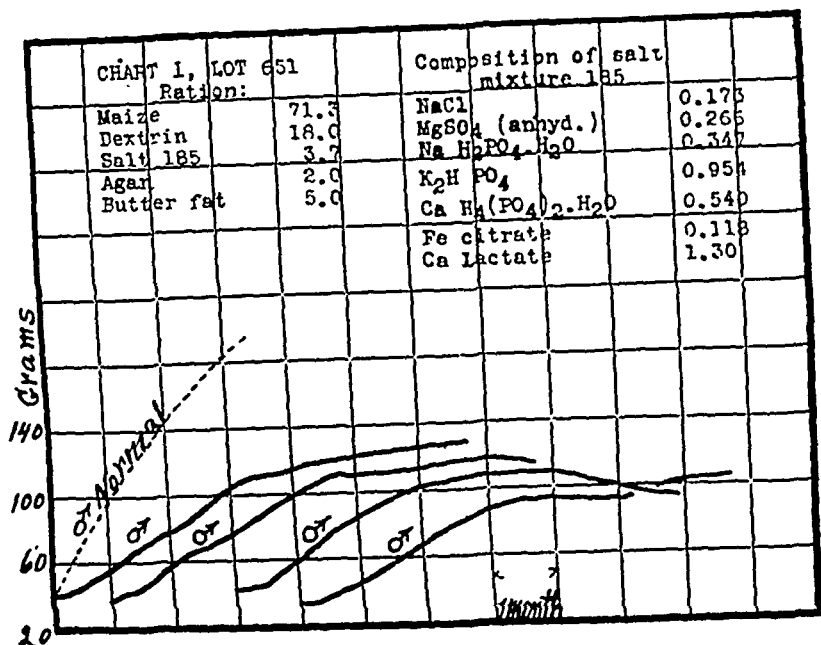


CHART 1. Lot 651 shows the growth curves of rats whose protein supply is limited to that contained in 71.3 per cent of ground maize. The salt mixture and butter fat included in the food mixture make satisfactory the inorganic content and the content of the fat-soluble A. The maize contains an abundance of the water-soluble B.<sup>6</sup> The one dietary factor which is responsible for the stunting and ultimate suspension of growth is the insufficient amount and poor quality of the protein in this food mixture. When 10 per cent of protein of wheat gluten is added to this diet it becomes adequate for normal growth (Chart 4). A similar improvement in growth should result in some degree from the addition of a single amino-acid, if we knew which of the essential ones is present in limiting amount in the mixture of maize proteins found in the seed. We have supplemented this ration with gelatin and with zein in order to ascertain whether lysine is the limiting amino-acid in the maize proteins (compare Charts 2 and 3).

<sup>6</sup> McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 153.

CHART 2, LOT -49

Ration.

Maize	71.3
Dextrin	8.0
Salt	1.7
Agar	2.0
Gelatin	10.0
Butter fat	5.0

LOT 659

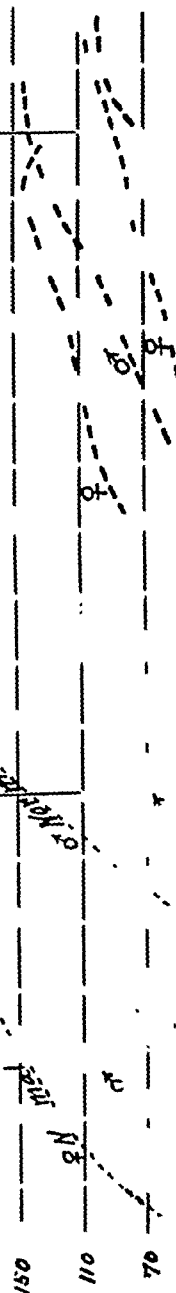
Rat on:

Maize	71.3
Dextrin	14.5
Salt	185
Agar	3.7
Gelatin	2.0
Butter fat	3.5
	5.0

For competition of salt

maize 185.0 Ch rt 1

Grams



30

CHART 2. Lots 659 and 649 illustrate that gelatin, when added to the proteins of the maize kernel, does not supply the essential amino-acid which is present in smallest amount and therefore the limiting factor. Gelatin is exceptionally rich in lysine (6 per cent). The results of these feeding experiments indicate that lysine is not the limiting factor which determines the biological value of the maize proteins. 3.5 per cent of gelatin supplies the same amount of lysine as does 10 per cent of wheat gluten proteins,<sup>7</sup> yet the latter addition greatly improves our standard ration in respect to its protein content (compare Charts 1 and 4), while the former is without beneficial effect. The rats in this group not only suffered stunting but also nearly complete loss of hair (Fig. 1). There were, however, no signs of an eczematous condition of the skin, which we have frequently observed, especially on the nose, ears, and tail of animals suffering malnutrition from

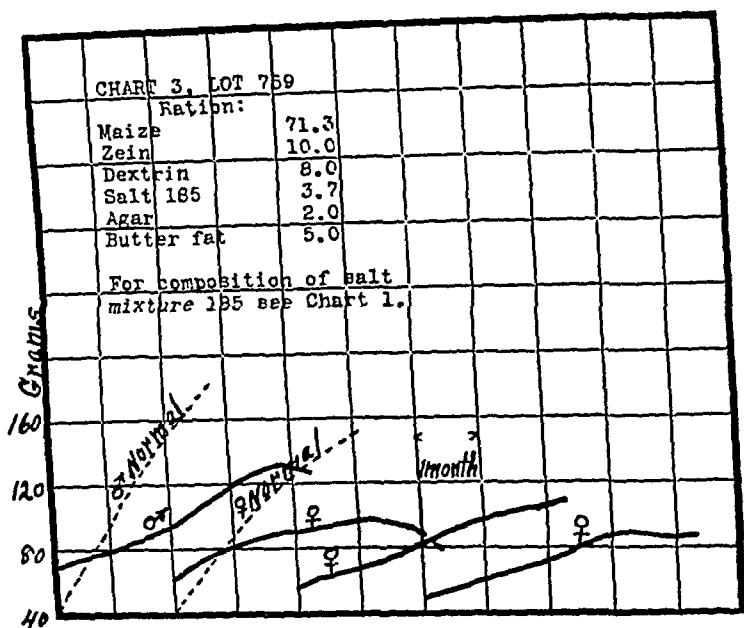


CHART 3. Lot 759 shows that the addition of zein to 7.13 per cent of maize proteins does not increase the biological value of the latter in a noticeable degree (compare Charts 1 and 3).

Zein contains no lysine. In marked contrast to its failure to supplement the proteins of the maize kernel is the pronounced benefit resulting from the addition of zein to a diet the protein content of which is inadequate in amount and which is wholly derived from the oat kernel (Chart 7).



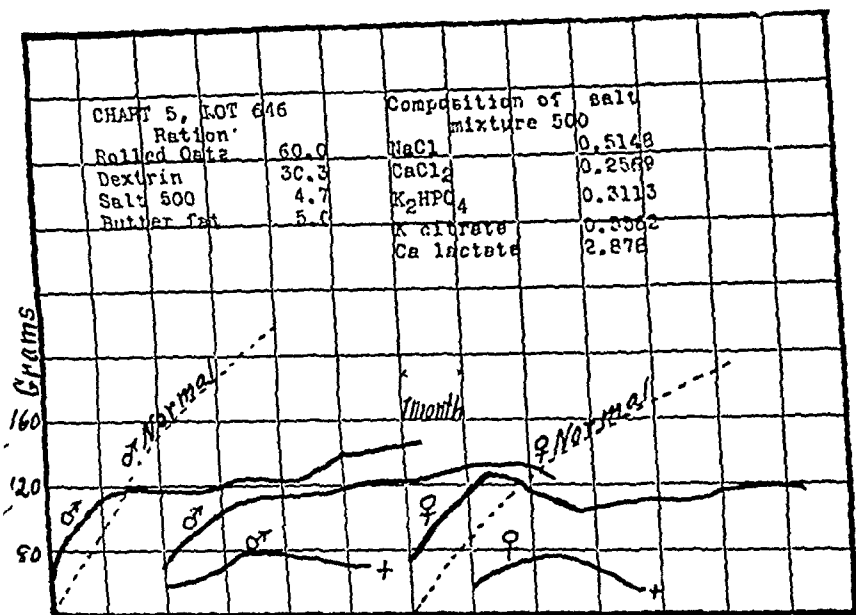


CHART 5. Lot 646 illustrates the character of the growth curves of rats fed a ration all the factors of which were properly adjusted except that the content of protein (9.0 per cent) which was derived solely from rolled oats was too low for normal nutrition. This food mixture, when a part of the dextrin is replaced by protein of suitable quality, becomes adequate for complete growth (Charts 6 and 7). We have selected this ration for the purpose of testing whether it is better supplemented by a protein yielding a high proportion of lysine (gelatin) than by one yielding low lysine (wheat proteins) or one yielding no lysine (zein). The results (Charts 6, 7, and 8) show that during the first 4 months the addition of zein led to greatly improved growth, despite the absence of lysine.

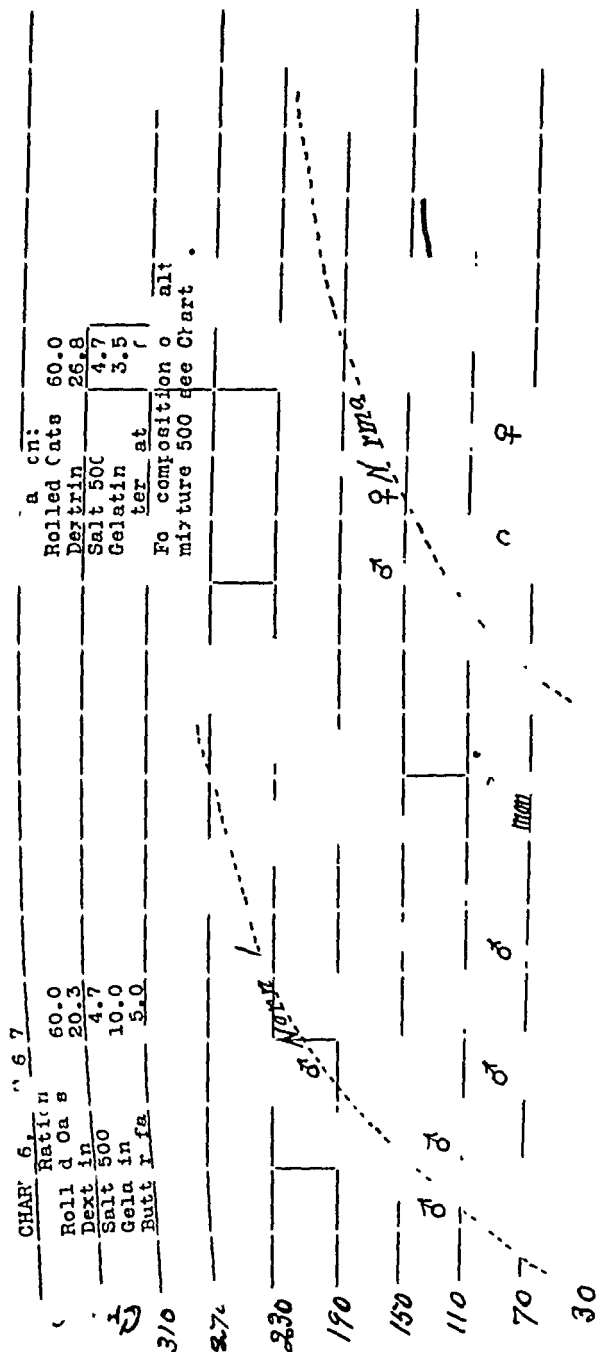


Chart 6. Lot 647 illustrates the fact that our standard mixture (Chart 5) with its 9 per cent of oat protein, becomes adequate to support growth at the normal rate to the full adult size when 10 per cent of gelatin is added. This serves as a remarkable illustration of the need of an understanding of the degree to which the proteins of one natural foodstuff make good the supply of the cleavage products of another which are present in smallest amount. Gelatin, as a source of proteins (compare Charts 5 and 6 with 1 and 2, and with 9, 11, and 12).

Lot 647 shows that the oat proteins are improved by the addition of 3.5 per cent of gelatin (compare Charts 5 and 6). This improvement is hardly to be attributed to the lysine content of the gelatin, since the addition of zein, which contains no lysine, likewise enhances the value of the oat protein (Chart 7) doubtless by supplying an additional amount of the essential amino-acid present in smallest amount in the proteins of the oat.

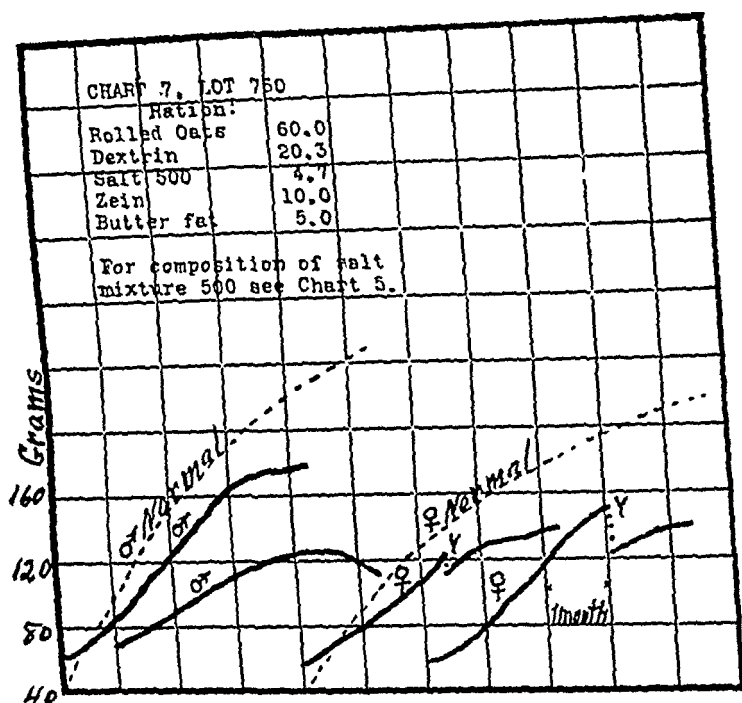


CHART 7. Lot 760 when compared with Lot 646 (Chart 5) shows the marked benefit to the animals which resulted from superimposing 10 per cent of zein upon the 9 per cent of oat protein contained in the basal ration employed in Charts 5 to 8 inclusive. On this ration with dextrin replacing the zein we have never seen rats grow beyond the 5th week. The addition of zein enabled them to grow at nearly the normal rate during 4 months and led to the production of two litters of young. The young were not reared. We interpret this result as leaving no doubt that a protein which does not yield lysine<sup>8</sup> serves to enhance the value of the proteins of the oat kernel, and seems to exclude the possibility of lysine being the limiting amino-acid in the protein mixture of the oat kernel.

<sup>8</sup> Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1913, xiv, 481.



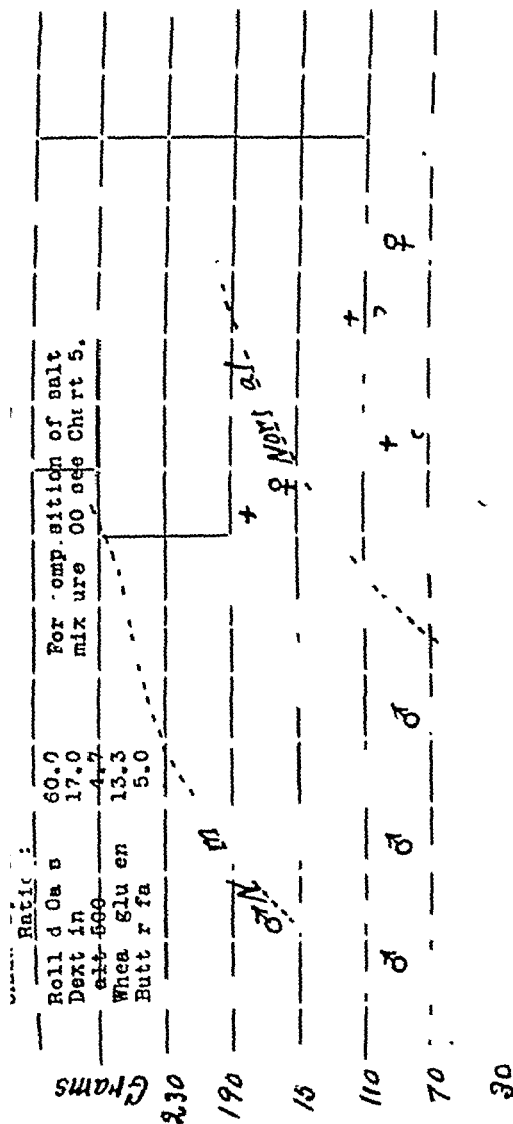


CHART 8. Lot 648 shows the result of superimposing 13.3 per cent of wheat gluten (equivalent to 10 per cent of protein) upon the oat proteins of our standard mixture (Chart 5). There is some improvement in the condition of the rats as a result of this addition as indicated by their ability to grow even to the 4th and 5th month, whereas without protein supplement the ration does not support growth beyond the 5th week (Chart 5). All the ten animals we have fed with the ration of Lot 648 have suffered suspension of growth at an early age, and the mortality in early life was high. We are inclined to believe that oats fed in liberal amount are injurious to rats. This we know to be true for wheat products\* and it would seem to be plausible that in the present instance there may be in some degree a supplementary relationship between the wheat and oat proteins whereby the latter are improved in character. There is a demonstrable injury due to the combined injurious action of the wheat gluten and oats.

\* Hart, E. B. McCollum, E. V., Steenbock, H., and Humphrey, G. C., Wisconsin Agric. Exp. Station, Bull. 17, 1911.

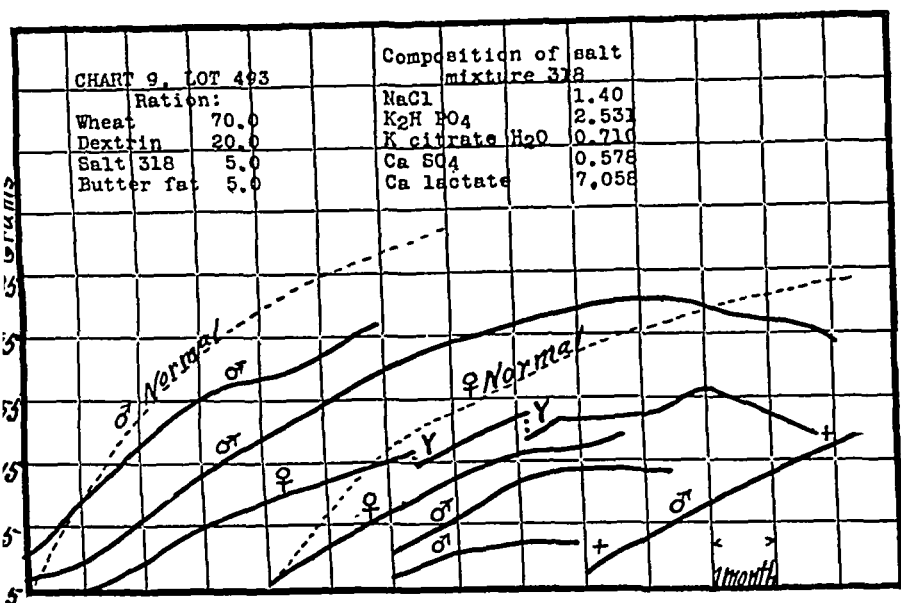


CHART 9. Lot 493 shows the degree of depression of growth in rats when the protein content of the diet is all derived from 70 per cent of wheat (7 per cent of protein). The substitution of a part of the dextrin of this food mixture by a suitable protein renders it adequate for the support of normal growth and reproduction.<sup>10</sup>

We have employed this mixture as a basal ration to which we have added (a) zein and (b) gelatin, to ascertain whether the supplementing action of these incomplete proteins would indicate whether lysine is the limiting amino-acid in the protein mixture in the wheat kernel (compare Charts 10, 11, and 12).

<sup>10</sup> McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 352.

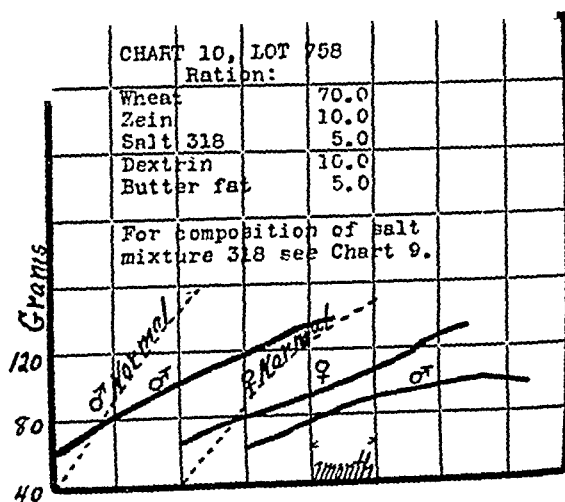


CHART 10. Lot 758 illustrates the failure of zein to supply the limiting amino-acids which determine the value of the wheat proteins. In this ration the addition of more of a protein from any source would lead to better growth, provided the protein added is qualitatively complete (i.e., contains all the essential amino-acids). An *incomplete protein* such as zein will improve the quality of the proteins only when it yields that amino-acid which is present in smallest amount in the proteins of the 70 per cent of wheat in the diet. Since among the indispensable amino-acids zein lacks only lysine and tryptophane, it is apparent that one of these is the limiting factor in the wheat proteins. Osborne and Mendel<sup>1</sup> have shown that for gliadin of wheat lysine is the limiting amino-acid.

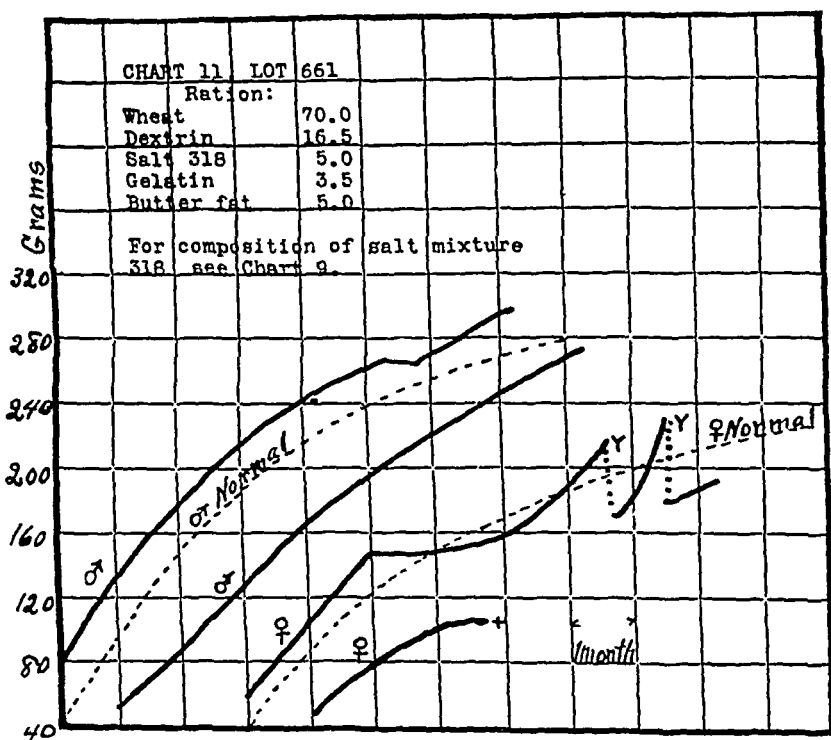


CHART 11. Lot 661 supports the idea that lysine is the limiting amino-acid which determines the value of the protein mixture of the wheat kernel. So small an addition as 3.5 per cent of gelatin, because of its high lysine content, greatly increases the utilizability of the wheat proteins. Without this addition growth takes place at a rate approximating half the normal expectation. With the gelatin addition growth proceeds at the normal rate (compare Charts 9 and 11).

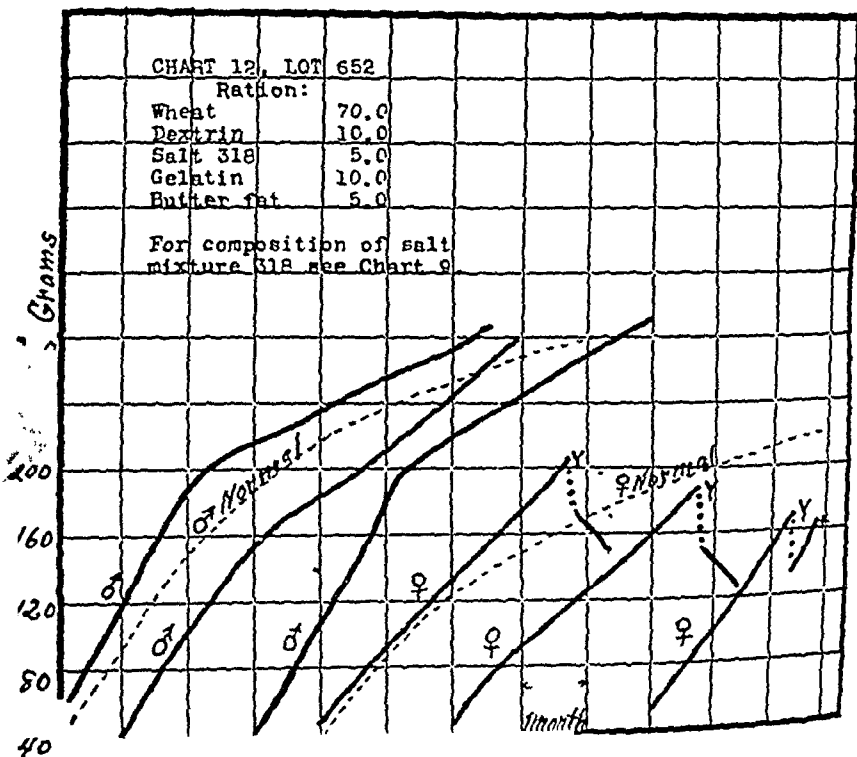


CHART 12. Lot 652 confirms the results obtained with Lot 661 (Chart 11), in showing that gelatin and the protein mixture of the wheat kernel mutually make good the shortage of certain amino-acids in the other and together form a protein mixture which is of much better quality than either component alone. This result is especially interesting when compared with the nutritive value of the proteins of the maize kernel with gelatin added (Charts 1 and 2). The latter combination is no better than the maize protein portion fed alone, and as is well known, gelatin fed as the sole protein is without value as a source of amino-acids. This forms one of the most striking illustrations yet observed of the importance of a knowledge of the supplementary relationship among the proteins of our foodstuffs. Fig. 3 shows the appearance of the rats fed on this mixture.

## EXPLANATION OF PLATE 4.

FIG. 1. These animals from Lot 649 (Chart 2) received a diet containing 17 per cent of protein, 10 per cent derived from gelatin and 7 per cent from maize. Gelatin does not improve the proteins of the maize kernel, even though it yields 6 per cent of lysine. This ration with wheat gluten or casein replacing the gelatin induces good growth and well-being. The rats were the same age as those in Fig. 2 when photographed.

FIG. 2. The rations of these two rats were identical except the source of the protein content. The rat on the left received 7 per cent of maize proteins plus 10 per cent of gelatin (Lot 649, Chart 2). The one on the right, from Lot 650 (Chart 4), received 7 per cent of maize proteins plus 10 per cent of wheat gluten protein. Gelatin supplements both wheat and oat proteins, but not those from maize. Both rats were the same age when photographed.

FIG. 3. This rat from Lot 652 (Chart 12) illustrates the good state of nutrition of animals fed wheat supplemented with gelatin. Wheat proteins are greatly improved by being combined with gelatin, whereas maize proteins are not (compare Charts 1 and 2 with 12). This rat is the same age as those in Figs. 1 and 2.









# THE EFFICIENCY OF CERTAIN MILK SUBSTITUTES IN CALF FEEDING.\*

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*(From the Laboratory of the Purdue Experiment Station, La Fayette, Ind.)*

(Received for publication, November 20, 1916.)

On account of the scarcity of skim milk for calf feeding an attempt was made to find some suitable substitutes. Experiments were carried on with a mixture of various vegetable proteins and also with a mixture of vegetable and animal proteins from various sources. The objects were, first, to determine to what extent a calf meal made up of both animal and vegetable feeding materials, rich in protein, could take the place of skim milk, and, second, to determine whether the proteins from wholly vegetable sources are capable of producing growth and development of the calf to the same extent as the proteins from animal sources.

The feeds were all mixed at the Purdue Dairy Barn, enough of each meal being mixed before the beginning of a period to carry through that period, which insured a uniform meal. A sample was taken from each meal at the time of mixing, for chemical estimation of the nitrogen content, and from this the amount of nitrogen consumed daily was calculated.

The skim milk which was fed was prepared by use of a cream separator and was fed fresh, at a temperature of about 98° F., or practically body temperature. The calf meals were mixed with water, 4 ounces of meal to 3 pounds of water at first, increasing to 12 ounces of meal with 9 pounds of water, and fed at the same temperature as the skim milk. The dry mash, consisting of

\* Acknowledgments are due to Professor O. F. Hunziker, Chief of the Dairy Department, for permission to carry on this investigation.

equal parts of ground corn and oats, was fed dry, the amount being carefully weighed to each calf, as was also the alfalfa hay. Salt and water were allowed *ad libitum*.

The calves were kept on as heavy a ration as possible, and were allowed all of each feed that they would readily consume. Whenever it was shown by portions of any feed left unconsumed that the amount given was too great, the rations were reduced accordingly. The care with which the calves were fed and the attending success can be shown by stating that only at two different times during the test was any calf off feed, and that only during one period of 3 to 5 days, in the case of two of these calves, was there a loss of body weight. In each case this loss was less than 1 pound each day during the time.

Feed refused at any time was weighed and a record made of the kind and amount.

#### FEED CHART.

##### *Feeding Periods and Feeds Received by Each Calf.*

Calf.....	B 40	B 43	B 41	B 44
Period I. 29 days.	Skim milk. Alfalfa hay. Dry mash.	Skim milk. Alfalfa hay. Dry mash.	Vegetable meal. Alfalfa hay. Dry mash.	Home mixed meal. Alfalfa hay. Dry mash.
Period II. 25 days.	Home mixed meal. Alfalfa hay. Dry mash.	Vegetable meal. Alfalfa hay. Dry mash.	Skim milk. Alfalfa hay. Dry mash.	Skim milk. Alfalfa hay. Dry mash.
Period III. 18 days.	Vegetable dried blood meal. Alfalfa hay. Dry mash.	Home mixed casein meal. Alfalfa hay. Dry mash.	Vegetable dried blood meal. Alfalfa hay. Dry mash.	Home mixed casein meal. Alfalfa hay. Dry mash.

The meals used were mixed as follows:

1. Vegetable meal.
 

Linseed meal.*	}	Equal parts by weight. N 4.97 per cent.
Soy bean meal.		
Cottonseed meal.		
Wheat middlings.		
2. Home mixed meal.
 

Hominy feed.	}	Equal parts by weight. N 5.60 " "
Linseed meal.*		
White Swan flour.		
Dried blood.		
3. Vegetable dried blood meal.
 

Soy bean meal.	}	Equal parts by weight. N 6.00 " "
Linseed meal.*		
Cottonseed meal.		
Wheat middlings.		
Dried blood.		
4. Home mixed casein meal.
 

Hominy feed.....	9 parts by weight.	}	N 5.02 " "
Linseed meal.*.....	9 " " "		
White Swan flour.....	9 " " "		
Casein .....	8 " " "		

Nitrogen content of the other feeds:

Skim milk.....	N 0.55	"	"
Mash.....	N 1.46	"	"
Alfalfa hay.....	N 2.53	"	"

\* Old process.

### *Average of Periods I, II, and III by Calves.*

The figures in the tables are obtained by taking an average of the figures by 3 day periods. These give the average daily nitrogen intake, nitrogen retained, total nitrogen excreted in urine, nitrogen excreted in the form of urea, nitrogen excreted in the feces, the body weight, the body gain, the weight of the urine, and the amount of dry matter or solids in the feces, each in gm., extending over a period of 24 hours.

Tables I and II give detailed illustrative figures selected for two of the four calves used in the feeding experiment.

Figs. 1 to 5 show the amount of nitrogen consumed and its distribution.

TABLE I.  
Average Daily Figures by 3 Day Periods.

Date.	Total nitrogen.						Weight of urine.	Dry matter in feces.	Body weight.	Gain.
	Intake.	In urine.	In feces.	Total excreted.	Retained.	Excreted as urea.				

*B 41. Period II. Skim Milk Ration.*

1916	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	kg.	kg.
Jan. 30.....	63.95	26.79	22.82	49.61	14.34	19.36	1,945*	714	78.9	1.63
" 31-Feb. 2..	63.87	20.67	22.54	43.21	20.65	15.17	1,673	712	80.0	1.08
Feb. 3-5.....	63.31	19.95	17.62	37.58	25.73	15.18	1,676	568	81.6	1.63
" 6-8.....	70.43	20.21	16.14	36.35	34.04	15.75	1,530	548	83.3	1.63
" 9-11.....	78.37	20.19	22.20	42.39	35.97	15.78	1,500	702	85.6	2.35
" 12-14.....	81.38	23.43	23.97	47.40	33.98	17.49	1,420	717	89.5	3.81
" 15-17.....	78.37	25.43	26.79	52.22	26.14	19.25	1,977	794	92.5	3.08
" 18-20.....	77.96	24.13	28.56	52.69	25.27	17.90	1,688	980	95.1	2.57
" 21-23.....	74.80	31.39	23.08	54.48	20.32	25.18	2,141	716	96.6	1.51

*B 41. Period III. Vegetable Dried Blood Meal Ration.*

Feb. 24-26.....	83.93	29.86	33.77	63.63	20.29	24.83	1,763	807	97.7	1.08
" 27-29.....	94.74	25.71	45.19	70.91	23.83	19.69	2,161	1,038	98.3	0.54
Mar. 1-3.....	84.36	20.81	47.23	68.05	16.31	15.34	2,348	1,049	101.4	3.08
" 4-6.....	96.55	23.90	45.32	69.22	27.32	18.19	1,971	912	105.5	4.17
" 7-9.....	96.55	26.94	48.90	75.85	20.69	22.99	2,760	1,040	108.4	2.90
" 10-12.....	96.55	26.33	47.36	73.70	22.84	21.99	2,316	1,032	111.4	2.94

*B 41. Period I. Vegetable Meal Ration.*

Jan. 1-2.....	44.78		19.50					471	60.1	
" 3-5.....	56.10	14.90	21.15	36.05	20.04	10.68	1,192	540	61.2	1.13
" 6-8.....	54.11	18.10	21.54	39.65	14.46	15.34	1,662	488	62.6	1.36
" 9-11.....	58.14	14.82	24.32	29.15	18.99	12.00	1,285	581	64.0	1.45
" 12-14.....	61.99	11.11	29.01	40.12	21.86	8.36	1,177.	721	66.1	2.08
" 15-17.....	59.13	19.13	27.47	46.60	12.52	14.67	1,723	658	68.3	2.17
" 18-20.....	63.09	19.34	31.27	50.61	12.48	13.79	1,629	778	70.5	2.17
" 21-23.....	64.97	19.28	25.23	44.52	20.45	14.61	2,238	697	72.7	2.17
" 24-26.....	65.28	19.93	27.06	46.99	18.29	15.67	2,205	760	74.9	2.20
" 27-29.....	68.41	20.17	26.64	46.81	21.60	16.70	2,054	761	77.3	2.41

\* Conhart, F. C., Du Bois, E. F., and Lusk, G., *J. Biol. Chem.*, 1916,

TABLE I—*Concluded.*

Date.	Total nitrogen.						Weight of urine.	Dry matter in feces.	Body weight.	Gain.
	Intake.	In urine.	In feces.	Total excreted.	Retained.	Excreted as urea.				

*B 44. Period II. Skim Milk Ration.*

1916	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	kg.	kg.
Jan. 30.....	60.70	17.72	30.26	47.99	12.71	15.18	2,458	742	71.7	1.79
" 31-Feb. 2...	57.78	17.93	22.57	40.50	17.27	12.41	1,618	637	72.5	0.87
Feb. 3-5.....	57.58	18.88	12.39	31.28	26.29	14.77	1,976	500	73.9	1.36
" 6-8.....	64.70	20.27	16.43	36.71	27.99	16.65	1,938	622	75.0	1.11
" 9-11.....	72.63	25.14	15.50	40.64	31.99	18.71	1,422	519	76.8	1.81
" 12-14.....	75.65	25.48	22.15	47.63	28.01	19.72	1,494	828	80.1	3.26
" 15-17.....	75.65	23.72	26.24	49.96	25.68	19.40	2,176	723	83.2	3.08
" 18-20.....	75.65	21.96	22.84	44.81	30.83	16.61	1,955	744	86.2	2.96
" 21-23.....	75.65	24.50	22.94	47.44	28.20	19.66	1,594	625	88.9	2.75

*B 44. Period III. Home Mixed Casein Meal Ration.*

Feb. 24-26.....	80.32	28.55	26.18	54.73	25.58	23.33	1,894	754	91.6	2.69
" 27-29.....	88.07	23.85	29.94	53.79	34.27	19.47	2,527	973	94.3	2.69
Mar. 1-3.....	79.75	28.23	33.74	61.97	17.77	21.85	2,619	834	96.9	2.60
" 4-6.....	89.87	25.66	35.65	61.31	28.56	20.50	2,767	875	99.3	2.44
" 7-9.....	88.67	27.58	35.23	62.81	25.85	22.75	2,522	1,062	101.8	2.45
" 10-12.....	89.87	28.25	35.74	63.99	25.87	24.14	2,990	929	104.3	2.54

*B 44. Period I. Home Mixed Meal Ration.*

Jan. 1-2.....	43.34		22.78					411	52.3	
" 3-5.....	55.91	11.67	19.13	30.80	25.10	8.46	974	428	54.2	1.81
" 6-8.....	56.55	13.88	25.51	39.40	17.15	10.73	1,515	544	56.0	1.81
" 9-11.....	56.57	13.44	26.51	39.95	16.62	10.53	2,149	601	57.6	1.57
" 12-14.....	58.05	11.94	22.11	34.05	23.99	9.43	1,992	509	58.7	1.14
" 15-17.....	57.09	15.01	27.80	42.82	14.27	10.69	1,269	626	60.4	1.72
" 18-20.....	60.25	16.62	25.16	41.79	18.52	12.69	1,823	703	63.4	2.99
" 21-23.....	60.38	15.43	25.14	40.58	19.80	11.44	1,505	590	65.5	2.08
" 24-26.....	60.90	15.15	28.98	44.14	16.75	11.71	1,956	739	67.3	1.75
" 27-29.....	65.37	15.72	26.36	42.09	23.27	12.37	2,593	699	69.9	2.61

TABLE II.  
Average Daily Figures for All Calves, by Periods.

Calf No.	Nitrogen.					Body weight.	Gain.	Weight of urine.	Dry matter in feces.	Ration
	Intake.	Retained.	In urine.	In feces.	Excreted as urea.					
Period I.										
B 40	56.13	25.51	15.21	15.39	11.81	68.3	2.09	1,135	453	Skim milk.
B 43	69.43	28.78	20.00	20.56	15.57	80.0	2.72	3,039	609	" "
B 41	61.25	17.85	17.42	25.96	13.54	68.6	1.91	1,685	665	Vegetable meal.
B 44	59.01	19.50	14.32	25.19	10.89	61.4	1.73	1,753	604	Home mixed meal.
Period II.										
B 40	81.63	25.38	18.79	37.45	14.27	88.1	1.87	2,051	827	Home mixed meal.
B 43	83.67	21.44	26.99	35.32	21.03	100.0	1.78	2,537	878	Vegetable meal.
B 41	73.56	27.76	23.17	22.61	17.71	88.0	2.21	1,701	717	Skim milk.
B 44	69.41	27.03	22.24	20.13	17.24	79.5	2.15	1,575	649	" "
Period III.										
B 40	91.68	19.89	24.42	47.31	19.46	104.0	2.47	2,143	996	Vegetable dried blood meal.
B 43	91.87	26.88	30.80	34.18	24.98	118.9	3.27	2,379	947	Home mixed case-in meal.
B 41	92.11	21.88	25.59	44.63	20.51	103.7	2.45	2,220	980	Vegetable dried blood meal.
B 44	86.09	26.32	27.02	32.74	22.01	98.0	2.57	2,553	905	Home mixed case-in meal.

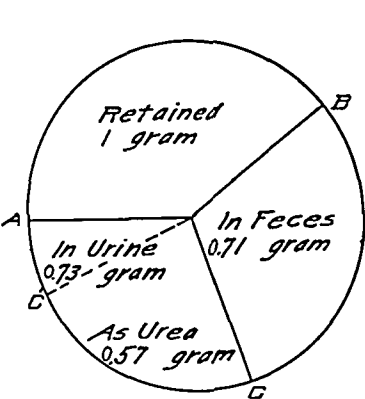


FIG. 1.

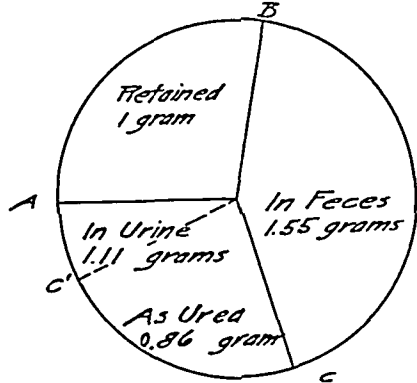


FIG. 2.

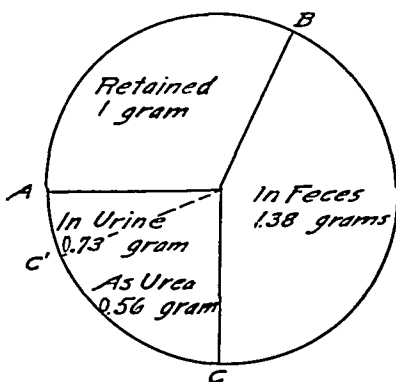


FIG. 3.

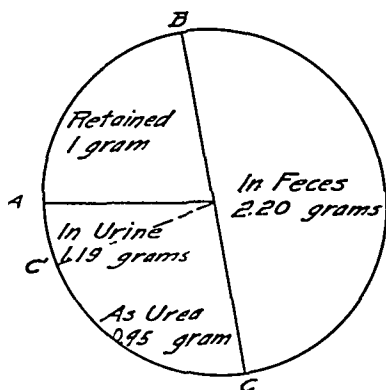


FIG. 4.

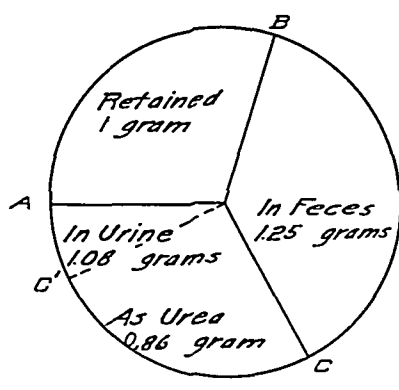


FIG. 5.

- FIG. 1. Skim milk ration. Circle = 2.44 gm.  
 FIG. 2. Vegetable meal ration. Circle = 3.66 gm.  
 FIG. 3. Home mixed meal ration. Circle = 3.11 gm.  
 FIG. 4. Vegetable dried blood meal. Circle = 4.39 gm.  
 FIG. 5. Home mixed casein meal. Circle = 3.33 gm.





FIG. 6. This crate shows one of the four used in the feeding experiment, and was moved outside of the barn for the purpose of being photographed.

#### SUMMARY.

I. The following amount of nitrogen consumed in the ration was retained.

	per cent
Skim milk ration.....	40.7
Home mixed meal.....	32.0
Home mixed casein meal.....	30.0
Vegetable meal.....	27.3
Vegetable dried blood meal.....	22.6

II. The excreted nitrogen was divided as follows:

	per cent
Skim milk ration: in urine .....	50.5
"    "    "    " feces .....	49.5
Home mixed casein meal ration: in urine.....	46.3
"    "    "    "    "    " feces .....	53.7
	41.2

	<i>per cent</i>
Vegetable dried blood meal ration: in urine.....	35.0
"      "      "      "      "      "      feces.....	64.9
Home mixed meal ration: in urine.....	34.2
"      "      "      "      "      "      feces.....	65.2

III. The gains in gm. of body weight per gm. of nitrogen consumed for different rations were:

	<i>gm.</i>
Skim milk.....	34.41
Home mixed casein meal ration.....	32.74
Vegetable dried blood meal ration.....	26.85
Home mixed meal ration.....	26.17
Vegetable meal ration.....	26.14

#### CONCLUSIONS.

1. The nitrogen intake was rather constant per kilo of body weight. The maximum difference was 12 per cent.

2. Less nitrogen was excreted from the vegetable ration than from the dried blood ration, there being a difference of 4.7 per cent.

3. It seems that when the nitrogen in the ration was the most suitable for growth, the nitrogen excreted was about evenly divided between the feces and the urine.

4. The total nitrogen excreted from each of the five rations indicates that the nitrogen in the skim milk ration was absorbed to the greatest advantage and the other feeds in the order named: home mixed meal, home mixed casein meal, vegetable meal, and vegetable dried blood meal.



# D-MANNOKETOHEPTOSE, A NEW SUGAR FROM THE AVOCADO.\*

By F. B. LA FORGE.

(From the Carbohydrate Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

(Received for publication, November 31, 1916.)

This article describes the isolation of a new ketose sugar, containing seven carbon atoms, from the fruit of *Persea gratissima*<sup>1</sup> commonly known as avocado or alligator pear. The sugar exists in the free state in the fruit and this fact is noteworthy because there is thus added another monosaccharide to the small number of such substances which have been found to occur in a free state in nature. It is striking that only two monosaccharides, namely, glucose and fructose, have been found widely distributed in any considerable quantity in the free state among all the numerous natural substances that have been investigated; the occurrence of other sugars in nature seems to be usually in combined forms, such as the complex sugars, polysaccharides and glucosides. The new sugar is both a ketose and a heptose and is accordingly the fourth natural ketose to be isolated, the other three being fructose, sorbose, and ketoxylose,<sup>2</sup> and is the first heptose to be found in nature, the previously known heptoses having all been prepared synthetically from hexoses.

The plant in which the new sugar occurs is also the source of the long known *d*-perseite,<sup>3</sup> a heptahydroxy alcohol which has been shown by Fischer<sup>4</sup> to be the alcohol obtained by the

\* Published with the permission of the Secretary of Agriculture.

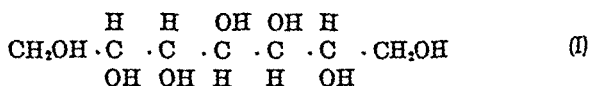
<sup>1</sup> My attention was first called to the presence of an unfermentable sugar in this fruit by Dr. E. E. Butterfield, then of Bellevue Hospital, New York.

<sup>2</sup> Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 319.

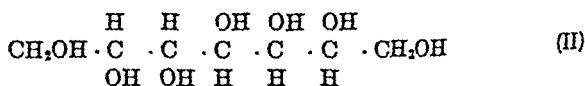
<sup>3</sup> Avequin, *Ann. chem. med. Ph. et Toxic*, 1831, vii, 464. Maquenne, *Compt. rend. Acad.*, 1888, cvii, 583.

<sup>4</sup> Fischer, E., and Passmore, F., *Ber. chem. Ges.*, 1890, xxiii, 2226.

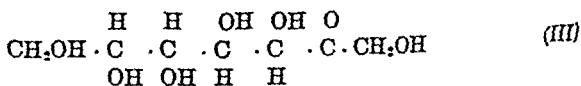
reduction of  $\alpha$ -mannoheptose. The occurrence in the same fruit of these two similar and rare seven carbon members of the sugar group suggests that there may be some biological relationship between them, and this view receives support from the fact that the new heptose can be transformed into *d*-perseite by reduction with sodium amalgam, as will be described later. There is also produced by this reduction a second crystalline alcohol which appears to be identical with *d*- $\beta$ -mannoheptite which Peirce<sup>5</sup> has lately prepared from *d*- $\beta$ -mannoheptose. In Peirce's article conclusive proof is advanced that *d*-perseite (*i.e.*, *d*- $\alpha$ -mannoheptite) has the configuration



and the corresponding *d*- $\beta$ -mannoheptite the configuration



While the formation from the new heptose of two alcohols having these space formulas may be taken to indicate that it has the configuration



it is to be remembered that the various alcohols of the sugar group are nearly impossible to distinguish by analysis, and in many cases have such similar properties that positive identification of them is a matter of considerable difficulty. It has, however, been possible to prove conclusively by other means that the avocado sugar has the configuration (III).

*Proof That the Avocado Sugar Is d-Mannoketoheptose, of Configuration (III).*

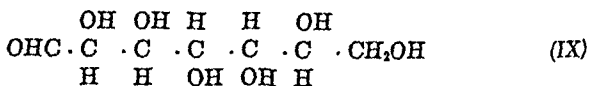
The *p*-bromophenylhydrazone of the new sugar was found to contain 20.8 per cent bromine, as an average of three closely

<sup>5</sup> Peirce, G., *J. Biol. Chem.*, 1915, xxiii, 327.

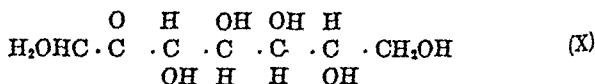
agreeing determinations. Since this hydrazone in the case of a hexose would contain 22.9 per cent bromine, in the case of a heptose 21.1 per cent, and in that of an octose 18.9 per cent, it is evident that the sugar is a heptose. This is in agreement with the fact previously mentioned that the reduction of the sugar yields a heptite, namely, perseite. The percentages of nitrogen and carbon in the *p*-bromophenylhydrazone, as recorded in the experimental part, also point to the same conclusion, which is further corroborated by the estimation of these elements in the phenylosazone of the new sugar. Its nitrogen content was 14.4 per cent as an average of several determinations, and the calculated value for a hexose osazone is 15.6, for that of a heptose 14.4, and in the case of an octose only 13.4. The carbon content was 59.2, and the corresponding values for the osazones of a hexose, heptose, and octose are 60.3, 58.8, and 57.4. This phenylosazone was found to melt at 200° and on comparing this value with the melting points of such of the heptose osazones as are known, it seemed probable that the substance might be identical with *d*-mannoheptose osazone, which Fischer<sup>4</sup> found to melt at about 200°. However, *l*-mannoheptose osazone would of course melt at the same temperature. Since the osazones of the *d* and *l* forms of mannoheptose must have rotations of equal magnitude but opposite sign, a measurement of this property can serve to distinguish the two forms and can also serve as additional evidence that the osazone of one of them is identical with that of the avocado sugar. The osazone of *d*-mannoheptose was prepared and found to rotate in the same direction (right) as the osazone of the avocado sugar, and solutions of the two substances, of equal strengths, in a mixture of pyridine and alcohol, gave the respective values, 0.35 and 0.48°, which do not differ beyond the limits of error. Dr. F. E. Wright in the following article has compared crystals of the two osazones by petrographic-microscopic methods, and finds the substances to be identical. It is, therefore, accepted that the osazone of the avocado heptose is identical with that from *d*-mannoheptose.

According to considerations of structure, *d*-mannoheptose osazone can be derived from three heptoses just as glucose osazone can be prepared from three hexoses, glucose, mannose, and fructose. One of these heptoses is, of course, *d*- $\alpha$ -mannoheptose which Peirce<sup>5</sup> has shown to have the configuration

which is the antipode of (VII). Neither (VII) nor (VIII) can be the configuration of perseulose because neither can give the configuration of *d*-perseite by reduction. The other *d*- $\beta$ -galaheptose of configuration



the antipode of (VI), was obtained by Fischer as an uncrystallizable sirup, whereas perseulose crystallizes readily. Hence, perseulose cannot be identical with either of these sugars and there remains the configuration



This conclusion is supported by Bertrand's generalization that the oxidation of the alcohols of the sugar group by *Bacterium xylinum* only takes place at the carbon atom in an  $\alpha$  position, and only then in case the hydroxyl on the  $\beta$  carbon atom is on the same side of the configuration as that on the  $\alpha$  carbon.

The conclusion that configuration (X) applies to perseulose requires that perseulose osazone be identical with *l*-galaheptose osazone, rather than with that from *d*-galaheptose, a deduction which is not obtainable alone from the identity of the melting points of the osazones. The osazone of *d*-galaheptose which melted at 222°, as mentioned, was found to rotate to the right. Hence, it must be concluded that *l*-galaheptose osazone, which seems to be identical with perseulose osazone, rotates to the left. The osazone of the avocado sugar rotates to the right, thus completing the evidence that the sugar itself cannot be identical with perseulose or with either of the *l*-galaheptoses.

In addition to *d*-mannoketoheptose and *d*-perseite the pulp of the avocado yielded another carbohydrate; namely, a gum insoluble in aqueous alcohol which gave on hydrolysis *l*-arabinose. The pentose was isolated in the form of its benzylphenylhydrazine.

It should be mentioned that no starch was found in the ripe fruit of the avocado.

In the following article are given crystallographic and optical measurements of mannoketoheptose and an optical comparison of the osazones of mannoketoheptose and mannoaldoheptose.<sup>9</sup>

#### EXPERIMENTAL.

##### *Preparation of d-Mannoketoheptose from the Avocado.*

The pulp of twelve avocados (Trapp variety), the aggregate weight of which was 3,500 gm., was passed through a sieve, mixed with about 6 liters of water, and filtered over night on folded filters. The residue was again extracted with the same amount of water and the combined filtrates were concentrated in a large dish on the steam bath to about 4 liters. The separated protein was removed by filtration and the solution concentrated under diminished pressure, with the addition of amyl alcohol to prevent foaming, to about 1.5 liters. 6 liters of about 98 per cent alcohol were added and the separated gum was filtered off with suction. The filtrate was then concentrated under diminished pressure to 250 to 300 cc. and sufficient absolute alcohol added to cause a permanent turbidity. After standing over night in the ice box, the perseite that had crystallized out was filtered off and the alcoholic solution was concentrated under diminished pressure to a thick sirup. It may be stated at this point that the first crystals of the sugar were obtained by preparing from such a sirup the crystalline *p*-bromophenylhydrazone, decomposing it with benzaldehyde and allowing the concentrated solution of the regenerated sugar to crystallize spontaneously. In later preparations it was found simpler to use the following procedure after crystals of the sugar had become available for seeding. The sirup referred to above was mixed with an equal volume of glacial acetic acid,<sup>10</sup> seeded with a few crystals of the sugar, and allowed

<sup>9</sup> These were made by Dr. F. E. Wright of the Geophysical Laboratory of this city, to whom I wish to express my thanks. I also wish to thank Dr. C. S. Hudson for revising the manuscript of this article and for helpful suggestions during the course of the work.

<sup>10</sup> According to the method of A. Wernicke, for crystallizing cane sugar (*Ber. chem. Ges.*, 1882, xv, 3105), and U. S. Patent No. 260,340 of June 27, 1882. Dr. I. K. Phelps was the first to use this method in the Bureau of Chemistry.



0.1375 gm. substance gave 0.2987 gm.  $\text{CO}_2$  and 0.0771  $\text{H}_2\text{O}$ .  
 0.1485 " " " 19.9 cc.  $\text{N}_2$  at 756 mm. and  $18^\circ$ .  
 0.1457 " " " 18.4 " " " 768 " "  $22^\circ$ .

	Calculated for $\text{C}_{10}\text{H}_{18}\text{O}_4\text{N}_2$ :	Found:
C.....	58.76	59.24
H.....	6.19	6.23
N.....	14.43	{ I. 14.55 { II. 14.38

A solution of 0.1000 gm. of osazone from the avocado sugar in 5 cc. of pyridine alcohol mixture rotated with sodium light in a 0.5 dm. tube after about 15 minutes  $+ 0.74^\circ$ , to the right, and after 24 hours  $+ 0.35^\circ$ .

A solution of 0.1000 gm. of osazone from mannoaldoheptose under the same conditions rotated after 15 minutes  $+ 0.78^\circ$ , to the right, and after 24 hours  $+ 0.48^\circ$ .

*Attempt to Oxidize the Avocado Sugar with Bromine.*—An aqueous solution of the sugar, 5 cc. of which corresponded to (I) 0.0964 gm. of  $\text{Cu}_2\text{O}$ , (II) 0.0954 gm. of  $\text{Cu}_2\text{O}$ , was allowed to stand for 4 days with an excess of bromine. After this time 5 cc. were boiled to remove the bromine and corresponded to 0.0952 gm. of  $\text{Cu}_2\text{O}$ , which shows that no appreciable oxidation had taken place.

*Reduction of Heptose.*—6.5 gm. of the avocado sugar were reduced with sodium amalgam in the usual way. The solution was kept at about  $0^\circ$  for the first 4 hours and neutralized with sulfuric acid frequently throughout the reaction. After about 8 hours' action of the amalgam, the solution which then showed no reduction with Fehling's solution was concentrated under diminished pressure until a large part of the sodium sulfate had crystallized out. It was then poured into 1.5 liters of 95 per cent alcohol and the alcoholic solution concentrated to about 15 cc. and diluted with about 200 cc. of absolute methyl alcohol. On standing 24 hours, 1.25 gm. of *d*-perseite crystallized out. This was once recrystallized from dilute methyl alcohol and it melted then at  $188^\circ$ . It rotated in saturated aqueous borax solution as follows:

$$[\alpha]_D^{20} = \frac{+ 0.41^\circ \times 5.6062}{1 \times 0.5069} = + 4.53^\circ$$

The characteristic heptacetate of *d*-perseite was obtained by warming one part of the crystals with ten parts of acetic anhydride in the presence of a trace of zinc chloride. The reaction mixture was poured into water and after the oil which first separated out had crystallized, the product was filtered off and purified by recrystallization from 70 per cent alcohol. It melted at 119°.

0.1526 gm. substance gave 0.0831 gm. H<sub>2</sub>O and 0.2806 gm. CO<sub>2</sub>.

	Calculated for C <sub>21</sub> H <sub>32</sub> O <sub>11</sub> :	Found:
C.....	49.80	50.14
H.....	5.92	6.05

From the filtrate from perseite the second heptite was obtained on evaporation as a sirup which crystallized on slow addition of alcohol and stirring. After washing with 95 per cent alcohol and drying, the yield amounted to 2.6 gm. This second hexite was recrystallized three times from fifteen to twenty parts of 95 per cent alcohol, and it melted at about 140° to a turbid viscous liquid which did not become clear until the temperature was raised to about 215°. It was, therefore, sublimed in a high vacuum and was then found to melt simultaneously with a sample of *d*-β-mannoheptite prepared by Peirce, at 149–155°, uncorrected.

0.1425 gm. substance gave 0.0952 gm. H<sub>2</sub>O and 0.2071 gm. CO<sub>2</sub>.

	Calculated for C <sub>7</sub> H <sub>12</sub> O <sub>7</sub> :	Found:
C.....	39.60	39.63
H.....	7.64	7.42

In aqueous solution it showed a dextrorotation as follows:

$$[\alpha]_D^{20} = \frac{+0.24^\circ \times 5.4987}{1 \times 0.5174} = +2.55^\circ$$

0.4994 gm. in 5 cc. of saturated aqueous borax solution rotated in a 1 dm. tube + 0.93°.

*d*-Galaheptose Osazone.—This was prepared in the usual way from *d*-α-galaheptose.<sup>8</sup> It crystallized from methyl alcohol in long needles which melted at 216° (222° corrected). A solution of 0.1 gm. of substance in 10 cc. of pyridine alcohol mixture

also the results obtained by computation from the weighted measurements of the angles:  $001:\bar{1}01 = 38^{\circ}33'$ ;  $100:\bar{1}\bar{1}0 = 52^{\circ}31'$ ;  $001:100 = 63^{\circ}24'$ .

The forms  $001$ ,  $100$ ,  $110$ ,  $\bar{1}\bar{1}0$ ,  $\bar{1}01$  were present on all five crystals; the forms  $111$ ,  $\bar{2}01$  were observed only once; the face  $111$  was so rounded and poorly developed that it cannot be considered to be an established form. The faces  $110$ ,  $\bar{1}\bar{1}0$  were invariably rounded and gave, on the goniometer, light streak signals whose general directions were in accord with the monoclinic sphenoidal symmetry of the crystal; the faces  $\bar{1}\bar{1}0$ ,  $\bar{1}\bar{1}0$ , on the other hand, were commonly flat and gave single, sharp re-

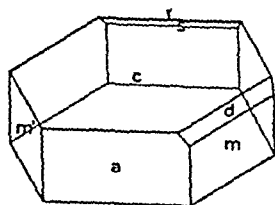


FIG. 1.

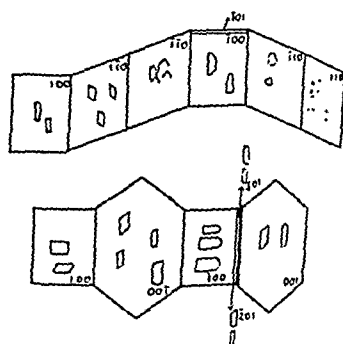


FIG. 2.

FIG. 1. Crystal of mannoketoheptose.

FIG. 2. Etch figures on different faces of a crystal of mannoketoheptose.

flection signals. This pronounced difference in the character of development of the prism faces on opposite sides of the plane of symmetry indicates that there is a digonal polar axis of symmetry, but not a plane of symmetry, present; this would place the crystal in the monoclinic hemimorphic class (digonal polar type) with the  $b$ -axis as the digonal axis of symmetry.

To test this inference a number of the crystals were immersed for 10 seconds in 95 per cent alcohol and etch figures produced. The etch figures were small and unequally developed on the different faces; they were studied both in reflected and in transmitted light. Some of the more commonly observed shapes are indicated in Fig. 2. These prove that there is no plane of sym-

metry present; also that the flat prism faces  $\bar{1}\bar{1}0$ ,  $\bar{1}\bar{1}0$  react, with respect to the solvent, differently from the faces  $110$ ,  $\bar{1}10$ . These relations suffice to establish the polar character of prism faces.

The optical relations are in accord with the monoclinic symmetry deduced from the crystallographic data. The optical orientation is:  $b = \beta$ ;  $c : \gamma = 26^\circ 36'$ . Optic axial angle  $2E > 180^\circ$ ; optical character +. The refractive indices were measured in sodium light with an Abbe-Pulfrich total refractometer on the flat basal pinacoid of a crystal about 1 sq. mm. in area.  $\alpha = 1.547$ ,  $\beta = 1.570$ ,  $\gamma = 1.595$ ;  $\gamma - \alpha = 0.048$ ,  $\gamma - \beta = 0.025$ ,  $\beta - \alpha = 0.023$ . The acute bisectrix  $\gamma$  is normal to the basal pinacoid; this was proved by direct observation of the interference figure from the basal pinacoid; also by measurement of the optic axial angle on the axial angle apparatus; and by the straight boundary line of the refractive index  $\gamma$  as observed on the total refractometer during a complete rotation of the crystal about the vertical axis.

The optic axial angle was measured directly on a small crystal immersed in a refractive liquid of index  $\beta = 1.570$ ; an average value of  $2V = 89^\circ$  was obtained. No appreciable dispersion was noted either of the bisectrices or of the optic axes. One of the optic axes on each side of the crystal presented an abnormal appearance as though there were two optic axes, the one directly under the first; the zero isogyre of this optic axis was inclined at an appreciable angle with the principal Nicol planes. The phenomena were, however, indistinct because of the smallness of the crystal, and this interesting aspect of the optic axis was not investigated further. The optic axial angle was also computed from the refractive indices and the value  $2V = 89^\circ$  obtained; the excellent agreement between this computed value and the above measured value is the result rather of chance than of precision in the measurements. It is significant, however, that in both cases  $\gamma$  is found to be the acute bisectrix; the crystals are, therefore, evidently optically positive.

*The Osazones of Mannoketoheptose and Mannaldoheptose.*

The two preparations consist, as far as can be determined, of the same crystal substance. They are lemon-yellow powders very fine-grained, and more or less aggregated in loose clusters

Under the microscope one of the samples is seen to consist of fine radial spherulites averaging 0.04 mm. in diameter. The second is composed chiefly of fine needles 0.01 to 0.02 mm. long and 0.002 to 0.005 mm. wide; in it fan-shaped portions of radial spherulites are also present in small amounts. The needles of both samples are strongly refracting, the average refractive index being approximately 1.775. The birefringence is medium. Especially characteristic are the abnormal interference colors which range from orange-yellow to blue-green and are the result of remarkably large dispersion of the bisectrices and also of strong spectral absorption of all light waves below 470  $\mu\mu$ . A measurement of the amount of dispersion was made with the aid of a monochromatic illuminator. The extinction angle on one of the needles varied with the wave length as follows:

Wave length in $\mu\mu$ :	616	577	552	514	495
Extinction angle ( $c : \alpha'$ ) :	15°	25.5°	34°	44°	50°

Below 490  $\mu\mu$  the crystals absorb the light so strongly that at 470  $\mu\mu$  practically no light is transmitted even in the thinnest needles. This fact was tested further by observing that the needles illuminated by the blue and violet lines (435, 407, 404  $\mu\mu$ ) of an intense quartz mercury arc appeared perfectly black and opaque.

As a result of the strong dispersion of the bisectrices the needles do not show in white light a position of total extinction but only a rapid change in hue from orange-yellow to blue-green at the position of normal extinction. The elongation of the fibers is positive ( $\gamma'$ ).

These optical data suffice to prove that the crystal system of this substance is either monoclinic or triclinic.

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